Ras Signaling through the RalGEF-Ral Pathway in *C. elegans*

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ABSTRACT

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(Under the direction of Channing J. Der)

The classical Ras effector pathway involves activation of the Raf-MEK-ERK mitogen-activated protein kinase cascade. Recent studies show that a second Ras effector cascade, Ral guanine nucleotide exchange factor (RalGEF) activation of the Ras-like (Ral) small GTPases, also promotes tumorigenic, invasive and metastatic cancer cell growth. How RalGEF-Ral downstream effector signaling facilitates Ras activity in cancer cells remains poorly defined. Studies of the *C. elegans* Ras ortholog have provided critical clues for delineating Ras signaling in mammalian cells. Components of Raf and RalGEF effector pathways are conserved in *C. elegans*, but only Raf is known to promote Ras function in vulval fate induction. Vulval precursor cell fates are patterned through activation of the epidermal growth factor (EGF)-EGF receptor (EGFR)-Ras-Raf-MEK-ERK cascade to specify the 1° fate, followed by Notch signaling to specify the 2° fate. Recently, the Raf pro-1° signal was also shown to be transiently active in presumptive 2° cells with unknown consequences. My studies have focused on determining the role of *C. elegans* RalGEF and Ral in Ras-dependent vulval patterning, with the long-term goal of understanding the *in vivo* function of Ral in mammals. We found that Ras signaling through RalGEF-Ral antagonizes pro-1° Ras-Raf signaling in parallel with or

downstream of the Ras-Raf signal. We showed that Ral regulates the balance of 1° and 2° fates, and contributes to EGF and Notch pro-2° activities. Ral expression was also found to be restricted to presumptive 2°s following initial induction. These results suggest that while Ras signals through Raf in 1° cells, in 2° cells Ras effector usage is switched to RalGEF-Ral. Thus, by such effector switching in presumptive 2° cells, the EGF signal transduced by Ras promotes a 2° fate instead of a 1° fate. From mammalian studies, we know that differential Ras effector usage exists, and can impact the efficacy of pharmacological inhibitors of Ras effector signaling currently under clinical trial evaluation. Our study provides insight into how cells spaced across gradients discriminate signal strength, and suggests that effector switching provides a mechanism by which the relative signal strength of two effector signals leads to distinct cellular outcomes.

“Do not try to bend the spoon. That’s impossible.

Instead, only try to realize the truth: there is no spoon.”

~ The Matrix

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TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

LIST OF ABBREVIATIONS

AC	Anchor Cell
ATP	Adenosine triphosphate
CAAX	C=cysteine; A=aliphatic amino acid; X=terminal amino acid
CDC25	Cell division cycle 25
cDNA	Complementary deoxyribonucleic acid
C.e.	*Caenorhabditis elegans*
C. elegans	*Caenorhabditis elegans*
CFP	Cyan fluorescent protein
CSL	CBF1, suppressor of hairless
C-terminal	Carboxy-terminal
d	Dominant
DIC	Differential interference contrast
dn	Dominant negative
DNA	Deoxyribonucleic acid
D.m.	*Drosophila melanogaster*
D. melanogaster	*Drosophila melanogaster*
DSL	Delta/Serrate/LAG-2
e.g.	Exempli gratia; for example
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELK-1	ETS like 1
ERK	Extracellular signal regulated kinase

ETS	E-twenty six (transcription factor)
FDA	Food and drug administration
FTase	Farnesyltransferase
FTI	Farnesyltransferase inhibitor
FTS	S-trans-trans-farnesylthiosalicylic acid
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
gf	Gain of function
GFP	Green fluorescent protein
GGTase-I	Geranylgeranyltransferase-I
GGTI	Geranylgeranyltransferase-I inhibitor
Grb2	Growth receptor-bound protein 2
GST	Glutathione-*S*-transferase
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HDAC	Histone deacetylase
HEK	Human embryonic kidney
HOM-C	Homeotic complex
Hox	Homeobox
H.s.	*Homo sapiens*
H. sapiens	*Homo sapiens*
HVR	Hypervariable region

Icmt	Isoprenylcysteine-O-carboxyl methyltransferase
L1	Larval stage 1
lf	Loss of function
lst	Lateral signaling target
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated extracellular signal regulated kinase
MKP	Mitogen-activated protein kinase phosphatase
mRNA	Message ribonucleic acid
Muv	Multi-vulva
NEXT	Notch extracellular truncated
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NICD	Notch intracellular domain
N-terminal	Amino-terminal
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PLCε	Phospholipase C epsilon
PLD-1	Phospholipase D1
Pn.p	Posterior daughters of ventral neuroectoblasts
PP2A	Protein phosphatase 2A

PTEN	Phosphatase and tensin homolog
RA	Ras association
Ral	Ras-like protein
RalBP1	Ral-binding protein 1
RalGDS	Ral guanine nucleotide dissociation stimulator
RalGPS	Ral GEFS with PH domain and SH3-binding motif
Rap	Ras-proximate
RBD	Ras binding domain
Rce1	Ras converting enzyme 1
REM	Ras exchange motif
rf	Reduction of function
RGL	Ral guanine nucleotide dissociation stimulator-like
Rheb	Ras homolog enriched in brain
Rin	Ras-interaction/interference protein
RNA	Ribonucleic acid
RNAi	RNA interference
SH3	Src homology 3
SMRT	Silencing mediator for retinoid and thyroid hormone
Sos	Son of sevenless
STAT3	Signal transducer and activator of transcription 3
TACE	TNF-α-converting enzyme
TANK	TRAF family member-associated NF-κB activator
TBK-1	TANK-binding kinase 1

TCF	T-cell factor
TF	Transcription Factor
Tiam1	T-cell lymphoma invasion and metastasis 1
TNF-α	Tumor necrosis factor alpha
TRAF	TNF receptor associated factor
ts	Temperature sensitive
TSC	Tuberous sclerosis complex
VPC	Vulval precursor cell
Vul	Vulvaless
WT	Wild type
ZONAB	ZO-1 associated nucleic acid-binding protein

CHAPTER 1: INTRODUCTION

I. Overview

Activating mutations in the Ras oncogene occur in 30% of all human cancers. Ras proteins utilize multiple downstream effectors in human oncogenesis with the Raf serine/threonine kinases, phosphoinositide 3-kinases (PI3K), and Ral-specific guanine nucleotide exchange factors (RalGEFs) being the best studied. While Ras activation of Raf and the mitogen-activated protein kinase (MAPK) cascade are well characterized in cancer, recent studies suggest that Ras signaling through the RalGEF-Ral small GTPase pathway also contributes significantly to Ras-mediated human cancer growth. However, the mechanisms by which RalGEF-Ral promote Ras-mediated oncogenesis are poorly defined. Characterization of critical Ras downstream effectors is key to unraveling the complexities of Ras-mediated oncogenesis. My dissertation research studies have focused on elucidating the RalGEF-Ral pathway in a simple *in vivo* system, *Caenorhabditis elegans* (*C. elegans*). In this introduction, I first provide an overview of Ras signaling and its role in human cancers. I then concentrate on the RalGEF-Ral effector pathway, and summarize the use of *C. elegans* as a model system for studying Ras pathway signaling.

II. Improved Therapies Needed for Pancreatic Cancer

In 2005 cancer surpassed heart disease to become the leading cause of death in the United States in people under the age of eighty-five (Jemal et al., 2005). A major challenge in the treatment of this disease is the molecular heterogeneity of cancer, both in genetic alterations and epigenetic changes. Thus, not all cancers

(even within each subtype) will respond to one therapeutic approach. Most conventional anti-cancer drugs target the highly proliferative nature of cancer cells (Chabner and Roberts, 2005). The main pitfall to these drugs is that not all cancer cells proliferate rapidly, resulting in some cancer types that are unresponsive to this treatment. These drugs also exhibit toxic side effects, as they cannot differentiate highly proliferative normal cells from neoplastic cells. In contrast to conventional anti-cancer drug design, current drug development has shifted towards targeted therapies (Gerber, 2008). These targeted therapies are designed to interfere specifically with the aberrantly functioning proteins and other molecules that drive the growth and development of cancer cells, thus potentially harming fewer normal cells and reducing toxic side effects. Eventually, the era of personalized medicine will begin where targeted therapies may allow physicians to individually tailor treatments based on the unique genetics of the patient's tumor.

Of the many cancer subtypes, pancreatic ductal adenocarcinoma (PDAC) is a particularly problematic disease that has limited treatment options and a five-year survival rate that remains lower than 5% (Hezel et al., 2006). PDAC is the most common pancreatic neoplasm and accounts for >85% of pancreatic tumor cases (Li et al., 2004; Warshaw and Fernandez-del Castillo, 1992). In the United States in 2009, around 42,500 new cases of pancreatic cancer were diagnosed and 35,200 individuals died of this disease (Table 1-1). Although pancreatic cancer accounts for only 2.5% of all new cancer cases, it is responsible for 6% of cancer related deaths each year. Therefore, pancreatic cancer has one of the highest fatality rates of all cancers and is the fourth leading cause of cancer related death in the United States

Table 1-1. 2009 U.S.A. Estimated Cancer Deaths*

Cancer Type	Number of Deaths	% Cancer Deaths
Lung & Bronchus	159,390	28
Colon & Rectum	49,920	9
Breast	40,610	7
Pancreas	35,240	6
Prostate	27,360	6
Leukemia	21,870	4
Non-Hodgkin Lymphoma	19,500	3
Liver & Bile Duct	18,160	3
Ovary	14,600	3
Esophagus	14,530	3
Urinary Bladder	14,330	3
All Sites	562,340	100

* Data from the American Cancer Society; (Jemal et al., 2009).

(Jemal et al., 2009). Since symptoms typically occur late, most patients are diagnosed with advanced metastatic disease making surgical resection impossible (Sohn et al., 2000). The current standard of care for patients with advanced PDAC is gemcitabine (a general cytotoxic drug), which provides only a modest improvement in median overall survival (5.65 versus 4.41 months) (Burris et al., 1997). Thus, the need to develop new treatments is clear. While we have made significant advances in our knowledge of the genetic events that underpin multi-step carcinogenesis, our understanding of how key signaling pathways interact in initiating and maintaining PDAC remains limited (Hezel et al., 2006). One signaling pathway that has received attention in cancer drug development is the Ras oncogene. Activating K-Ras mutations occur in more than 90% of pancreatic tumors (Almoguera et al., 1988; Smit et al., 1988), and are among the earliest detectable genetic changes (Caldas et al., 1994; Yanagisawa et al., 1993). The recent genome-wide sequencing of pancreatic cancer verified that K-Ras mutations represent the most frequently mutated gene in this cancer (Jones et al., 2008). While substantial experimental studies validate the importance of continued mutant K-Ras function for pancreatic cancer growth (Brummelkamp et al., 2002; Fleming et al., 2005; Hingorani et al., 2003; Lim and Counter, 2005), efforts to develop anti-K-Ras drugs have to date met with no success.

III. Ras Proteins and Their Role in Human Cancer

A. Ras Proteins as Molecular Switches

Ras is the prototypical member of a large superfamily of small GTPases (156 members) that share significant structural identity (30-55% amino acid identity) and biochemical activity (GTP binding and hydrolysis) (Wennerberg et al., 2005). Sequence and functional similarities divide the Ras superfamily into at least five major subfamilies: Ras, Rho, Rab, Arf, and Ran. In general, Ras family members (36 genes encoding 39 proteins; Figure 1-1) control cell growth and differentiation, Rho family members control actin cytoskeleton organization, Rab and Arf family members control intracellular vesicular transport, and the single Ran family member controls nuclear transport (Colicelli, 2004; Takai et al., 2001).

The three Ras genes encode four highly related proteins (H-Ras, N-Ras, K-Ras4A, and K-Ras4B; Figure 1-2) (Karnoub and Weinberg, 2008). Like other members of the family, Ras proteins function as regulated switches that cycle between inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP)-bound protein conformations (Figure 1-3) (Vetter and Wittinghofer, 2001). The intrinsic GDP/GTP exchange and GTP hydrolysis rates of Ras proteins are too slow for physiological reactions, and thus accessory proteins exist to accelerate this process (Vetter and Wittinghofer, 2001). In normal quiescent cells, Ras is GDP-bound and inactive. Upon activation, guanine nucleotide exchange factors (GEFs; e.g., Sos) stimulate the release of bound GDP (Chardin et al., 1993; Egan et al., 1993). GDP dissociation promotes the formation of active GTP-bound Ras since the cellular amounts of GTP exceed that of GDP (Scheele et al., 1995). During nucleotide exchange, the highly mobile regions of Ras, switch I

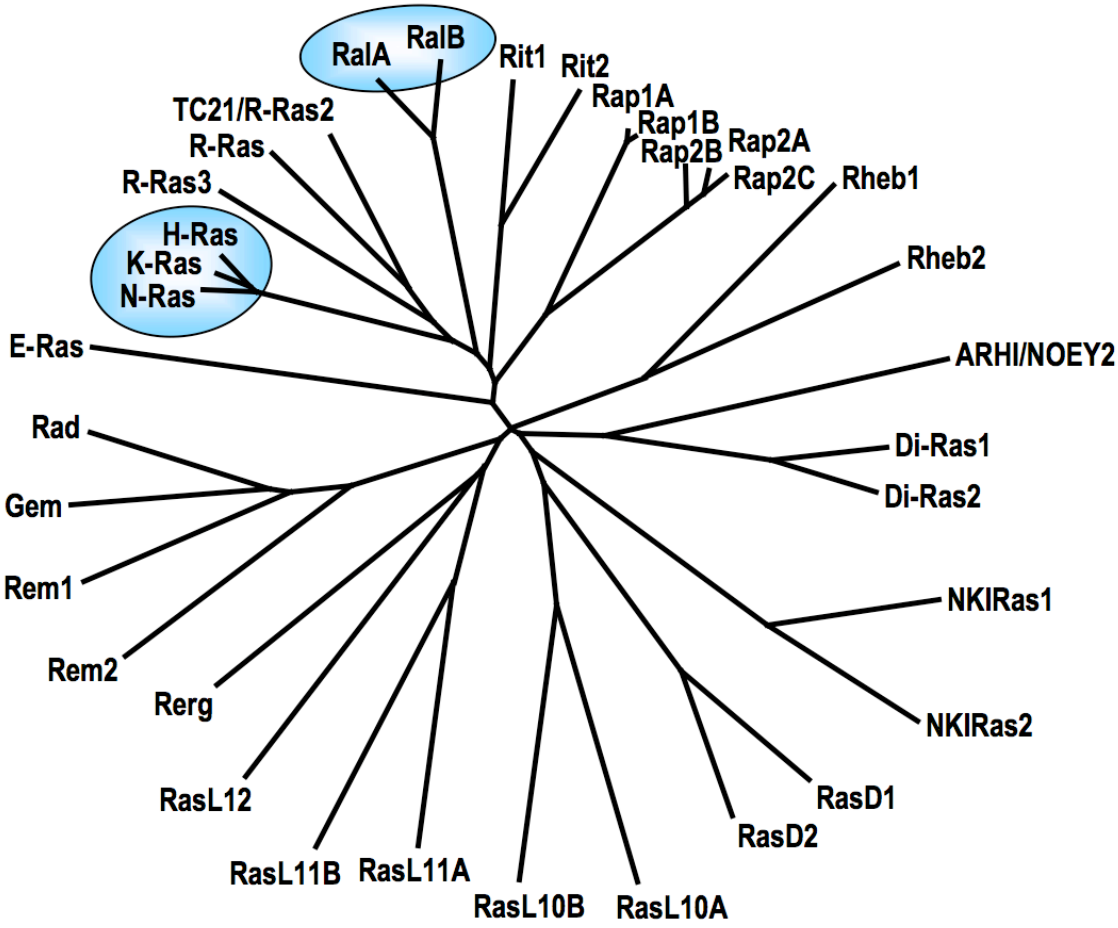


Figure 1-1. The Ras Family of Small GTPases.
The three Ras isoforms (H-, K-, and N-Ras) belong to the Ras family (shown here) of the Ras superfamily (including Rho, Rab, Arf, and Ran, not shown) of small GTPases. In this family, only the Ras isoforms and the Ral isoforms (RalA and RalB) have been implicated in the promotion of pancreatic cancer. The GTP-binding domain sequences of human Ras subfamily members were used to generate this dendrogram (ClustalX). K-Ras represents the K-Ras4B splice variant, the predominant variant expressed in human cells.

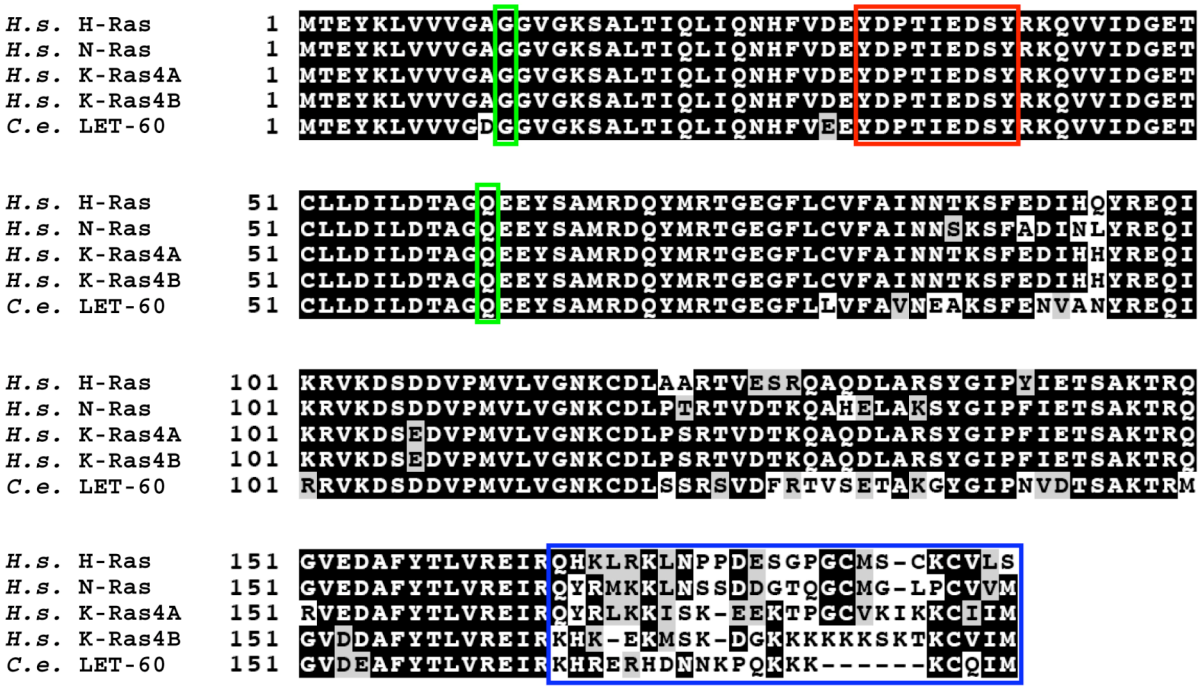


Figure 1-2. Alignment of Ras Orthologs.
ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used to align full-length protein sequences of *Homo sapiens* (*H.s.*) H-Ras (NCBI Accession NP_005334), *H.s.* N-Ras (NP_002515), *H.s.* K-Ras4A (NP_203524), *H.s.* K-Ras4B (NP_004976) and *Caenorhabditis elegans* (*C.e.*) LET-60 (NP_502213). Sequences were shaded using Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Greater than 50% identical or similar residues are marked with black or grey shading, respectively. The key residues for GAP stimulation of GTP hydrolysis (G12 and Q61) are boxed in green, the main core effector domain is boxed in red, and the C-terminal hypervariable and CAAX prenylation signal motif region in blue.

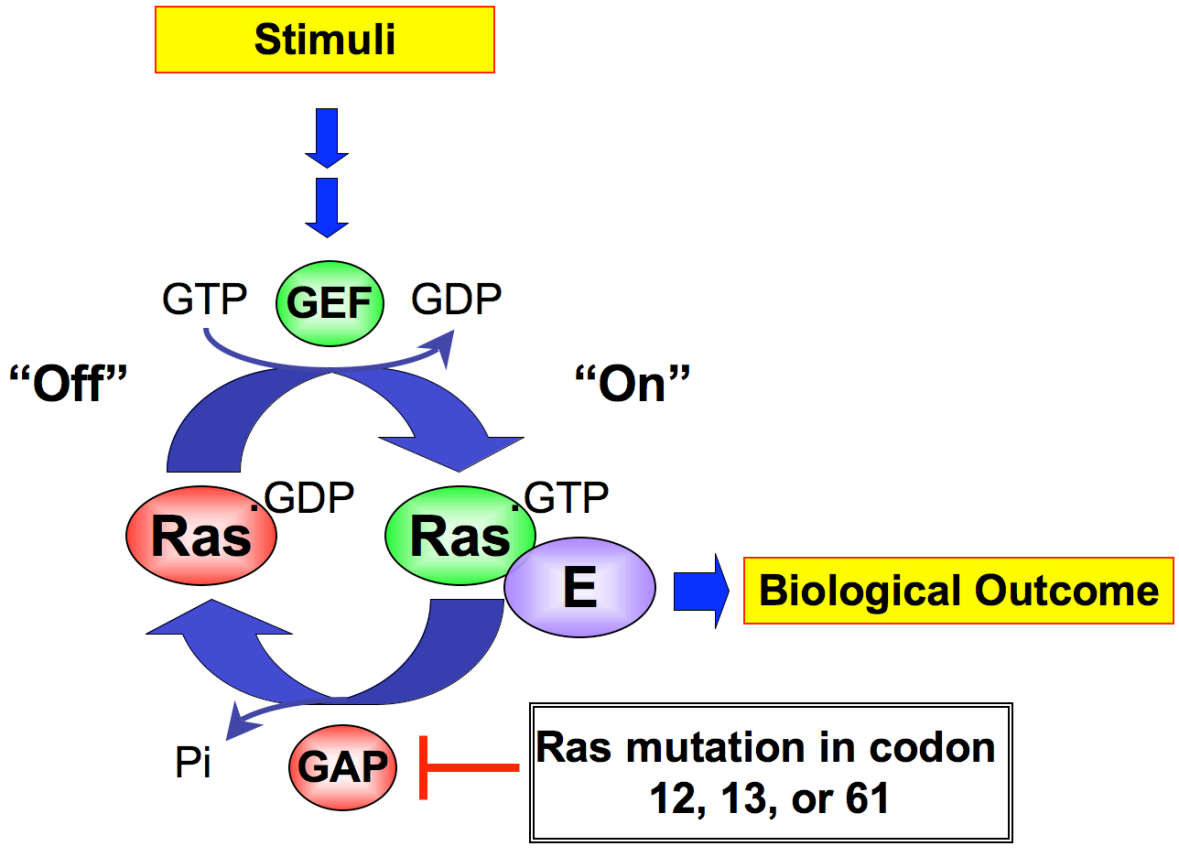


Figure 1-3. The GTPase Cycle.
Ras proteins function as regulated GDP/GTP switches. Diverse extracellular signals regulate the activity of guanine nucleotide exchange factors (GEFs), which promote GTP loading and activation of Ras. Ras-GTP binds to and activates downstream effectors (E) leading to biological outcomes. Ras signaling is arrested by GTPase activating protein (GAP) stimulated hydrolysis of bound GTP to GDP. Oncogenic Ras mutant proteins (with single amino acid substitutions, most commonly residues 12, 13, or 61) are GAP-insensitive and chronically GTP-bound and active.

(residues 30-38) and switch II (residues 59-76), undergo structural changes (Boriack-Sjodin et al., 1998; Lenzen et al., 1998). When Ras is GTP-bound, these structural changes increase the accessibility of the core effector domain (residues 32-40) resulting in high affinity binding to effector proteins. Thus once GTP-bound, Ras binds to a spectrum of downstream effector targets to control a diverse array of cellular processes including actin organization, cell survival, gene expression, cell cycle progression, and vesicular transport (Shields et al., 2000). Ras-specific GTPase activating proteins (RasGAPs) stimulate the intrinsic rate of GTP hydrolysis, returning Ras to an inactive GDP-bound conformation that has low affinity for effectors (Li and Zhang, 2004). Oncogenic Ras proteins contain single amino acid substitutions, most commonly at residues G12, G13 or Q61 that render the proteins impaired in intrinsic and GAP-stimulated GTP hydrolysis (Bos, 1989; Herrmann, 2003). Thus, oncogenic Ras proteins exist in a constitutively active GTP-bound conformation.

B. Post-translational Processing of Ras and Membrane Association

Ras requires association with cellular membranes for its biological activity (Wright and Philips, 2006). Though activated Ras most notably localizes to the plasma membrane, recent studies have shown additional localization to other membrane compartments including the Golgi, endoplasmic reticulum, and mitochondria (Bivona et al., 2006; Chiu et al., 2002; Choy et al., 1999). Proper subcellular localization and membrane association of Ras is achieved through a series of post-translational modifications initiated by enzymes that recognize the

CAAX (C=cysteine, A=aliphatic amino acid, and X=terminal amino acid) tetrapeptide motif present at the carboxyl-terminus of Ras (Figure 1-4) (Clarke, 1992). First, the enzyme farnesyltransferase (FTase) covalently adds a 15-carbon farnesyl isoprenoid lipid to the C-terminal cysteine residue (Casey and Seabra, 1996; Casey et al., 1989). Then the Ras converting enzyme 1 (Rce1) endoprotease cleaves the AAX residues (Boyartchuk et al., 1997; Otto et al., 1999) and finally isoprenylcysteine-O-carboxyl methyltransferase (Icmt) methylates the farnesylated cysteine residue (Clarke et al., 1988). In addition to the CAAX motif, Ras also requires a 'second signal' that resides in the hypervariable region (HVR) directly upstream of the CAAX sequence (Hancock et al., 1991). This 'second signal' is either cysteine(s) that can be modified by palmitoylation (H-, K-Ras4A and N-Ras) or a highly basic polylysine sequence (K-Ras4B) (Hancock et al., 1990). These modifications promote Ras membrane association by increasing the hydrophobicity of its C-terminus.

C. Ras Mutations in Cancer

Ras is mutationally activated in 30% of all human cancers (Bos, 1989), with pancreatic (90%), colorectal (50%), and lung (30%) carcinomas having the highest prevalence (Jones et al., 2008; Karnoub and Weinberg, 2008). Although the three Ras genes encode proteins with 90% identity at the amino acid level, they are mutationally activated at different frequencies in all human tumors: 3% (*H-Ras*), 8% (*N-Ras*) and 21% (*K-Ras*) (COSMIC). Additionally, the particular isoforms that are mutated also exhibit striking cancer type patterns. For example, H-Ras mutations

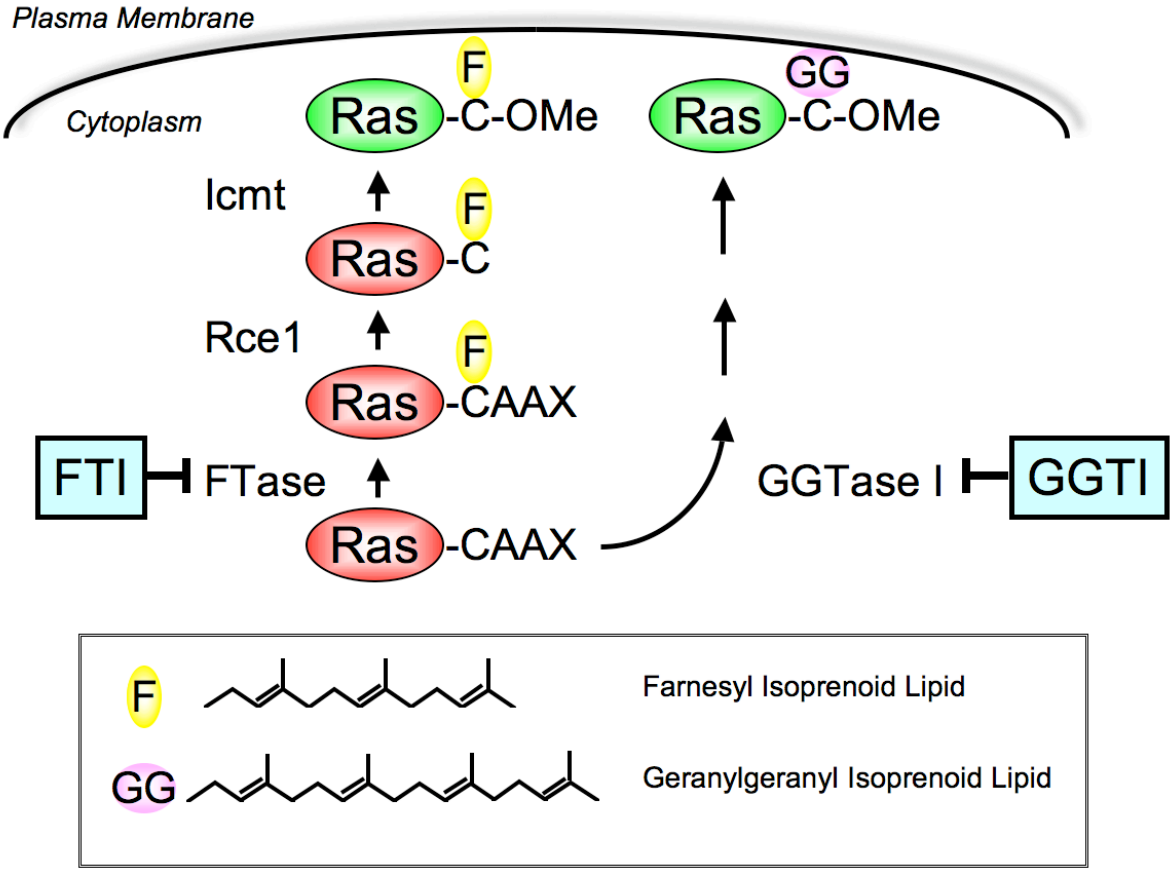


Figure 1-4. Post-translational Processing of Ras.
Ras requires post-translational modifications to achieve proper membrane association. FTase catalyzes covalent addition of a farnesyl isoprenoid lipid to the C-terminal cysteine residue of the CAAX motif (C=cysteine, A=aliphatic amino acid, and X=terminal amino acid). The Rce1 endoprotease then cleaves the AAX residues followed by carboxylmethylation of the now terminal farnesylated cysteine residue by ICMT. FTIs block the FTase and all other subsequent steps. In the presence of FTIs, K-Ras4B and N-Ras undergo alternative prenylation by GGTase-I, which adds a geranylgeranyl isoprenoid lipid to the C-terminal cysteine residue. GGTIs block GGTase-I activity. Not shown in this figure are the role of second signal C-terminal sequence elements required for full plasma membrane association: palmitoylation of C-terminal cysteine residues in H-Ras, N-Ras, and K-Ras4A and polybasic sequences for K-Ras4B.

have been found predominately in squamous cell carcinomas, N-Ras in leukemias and melanomas, and K-Ras in adenocarcinomas of the colon, lung and pancreas (Table 1-2) (Lau and Haigis, 2009).

D. Therapeutic Targeting of Ras

Despite the unequivocal importance of Ras in human cancer, it is not considered a tractable target for drug discovery, and therefore there are no direct Ras inhibitors currently in the clinic. After over three decades of efforts to develop anti-Ras therapies, Ras has proven to be an elusive drug target. Much of general targeted drug discovery has focused on blocking the low micromolar binding of adenosine triphosphate (ATP) to protein kinases. This strategy for kinase inhibition has proven successful as many FDA-approved protein kinase inhibitors are currently in the clinic (Sebolt-Leopold and English, 2006). However, it is not feasible to develop small molecules to inhibit the high picomolar affinity of Ras for guanosine nucleotides (John et al., 1990; Manne et al., 1984). Thus, current anti-Ras strategies have focused on indirect approaches to block Ras.

Since proper subcellular localization of Ras is critical for activation and effector binding, another anti-Ras strategy has been to prevent Ras association with the plasma membrane (Konstantinopoulos et al., 2007). Early anti-Ras strategies focused on developing FTase inhibitors (FTIs), as it had been shown that inhibition of the initial farnesylation step inhibits all subsequent processing steps (Gutierrez et al., 1989). However, FTIs have not proven to be effective anti-Ras agents, as the isoforms most commonly mutated in cancer (K- and N-Ras) undergo alternative

Table 1-2. Ras Mutations in Human Cancers*

Cancer Type	% K-Ras	% N-Ras	% H-Ras
Pancreatic Ductal Adenocarcinoma	71	1	0
Biliary Tract Adenocarcinoma	37	2	0
Colon Adenocarcinoma	36	3	0
Lung Large Cell Carcinoma	22	7	4
Chronic Myelomonocytic Leukemia	12	18	1
Melanoma (Skin)	2	21	1
Squamous Cell Carcinoma (Skin)	5	8	9

*Data is compiled from the COSMIC database; (Lau and Haigis, 2009)

prenylation (geranylgeranylation) that support Ras function and signal transduction when FTase activity is inhibited (Figure 1-4) (Cox and Der, 2002; Rowinsky, 2006).

Currently research efforts have shifted to other methods of disrupting Ras membrane association (Blum et al., 2008). The other CAAX-processing steps (Rce1 and Icmt) are being evaluated as therapeutic targets (Clarke and Tamanoi, 2004; Winter-Vann and Casey, 2005). Like FTIs, one major concern with blocking Rce1 or Icmt is the fact that the number of substrates for these enzymes is extensive (Reid et al., 2004). Thus compounds that inhibit CAAX-mediated post-translational processing may have toxic effects caused by inhibition of other CAAX-terminating proteins. Another approach for disrupting Ras localization is farnesyl isoprenoid-containing small molecules. These compounds are structural analogs of the farnesylated cysteine, and are thought to compete with farnesylated Ras for membrane binding sites (Blum et al., 2008; Rotblat et al., 2008). Currently two such compounds are undergoing Phase I/II clinical trials: S-trans-trans-farnesylthiosalicylic acid (FTS; salirasib) (Gana-Weisz et al., 1997) and TLN-4601 (formerly ECO-4601) (Gourdeau et al., 2008). Thus far both compounds have been found to be safe and well tolerated in patients. Encouragingly, salirasib has shown positive results against pancreatic cancer in Phase I/II clinical trials. In this study, salirasib in combination with gemcitabine almost doubled the mean survival of pancreatic cancer patients (10.8 versus 6.2 months on gemcitabine alone) (Laheru et al., 2009). Though these results are promising, inhibition efficacy of these compounds in specifically inhibiting patient Ras-driven tumors needs to be further evaluated.

Another indirect approach for targeting Ras-mediated oncogenesis is to inhibit downstream effector signaling. The GTP-bound state of Ras has high affinity for a spectrum of downstream effectors with the Raf serine/threonine kinases (c-Raf-1, A-Raf and B-Raf) being the best studied. Intense efforts have focused on developing inhibitors of the Raf-MEK-ERK pathway as possible anti-cancer agents (Roberts and Der, 2007). Although extensive experimental evidence supports the Raf-MEK-ERK cascade as a critical mediator of Ras-induced oncogenesis, recent studies have established that Ras also utilizes other effector signaling pathways to promote tumorigenesis (Figure 1-5) (Repasky et al., 2004). The complexity of signaling pathways mediated by Ras presents a challenge to effective blockade of Ras signaling in oncogenesis. Will inhibition of the Raf-MEK-ERK pathway be sufficient to effectively block Ras-mediated oncogenesis, or will inhibition of multiple effector pathways be required? Currently the therapeutic index of blocking a single Ras effector pathway is uncertain. Also, whether the Raf-MEK-ERK pathway is the most clinically relevant target for blocking Ras-mediated oncogenesis remains undetermined. The characterization of critical Ras downstream effectors will be key to unraveling the complexities of Ras-mediated oncogenesis and developing future anti-Ras strategies.

E. Ras Effector Pathways Important for Transformation

In 1992, a convergence of findings from studies in *C. elegans*, *D. melanogaster,* and mammalian systems delineated the first well-defined signal transduction pathway that connected cell surface signaling to the nucleus (Egan and

Weinberg, 1993). It is now understood that the simple linear EGFR-Grb2-Sos-Ras-Raf-MEK-ERK signaling cascade represents a core signaling pathway within a complex signaling network that involves signaling inputs and outputs at every level (Mitin et al., 2005). Currently there are at least ten distinct functional classes of Ras effectors identified. Five of these effector classes have demonstrated roles in Ras transformation: Raf serine/threonine kinases, class I p110 catalytic subunits of phosphoinositide 3-kinases (PI3K), Ral small GTPase-specific GEFs (RalGEFs), T-cell lymphoma invasion and metastasis 1 (Tiam1), and phospholipase C epsilon (PLCε) (Figure 1-5) (Repasky et al., 2004).

The Raf serine/threonine kinases are the most widely studied and best-characterized effectors of Ras function (Chong et al., 2003). Ras association with Raf initiates events, including plasma membrane translocation, that lead to the activation of Raf (Shields et al., 2000). Once activated, Raf phosphorylates and activates the MEK1 and MEK2 dual specificity kinases, which in turn phosphorylate and activate the ERK1 and ERK2 mitogen-activated-protein-kinases (MAPKs). Activated ERK then translocates to the nucleus where it phosphorylates transcription factors, including members of the ETS family (e.g., Elk-1), thereby causing gene expression changes that regulate cell growth and differentiation (Seth et al., 1992; Yordy and Muise-Helmericks, 2000). Substantial evidence from rodent fibroblast model cell systems supports the role of Raf as a key mediator of Ras-oncogenesis (Shields et al., 2000). The finding of mutationally activated B-Raf in a variety of human tumors further supports the importance of Raf in cancer (Davies et al., 2002). However, B-Raf mutations are rare in PDAC (Immervoll et al., 2006). Furthermore,

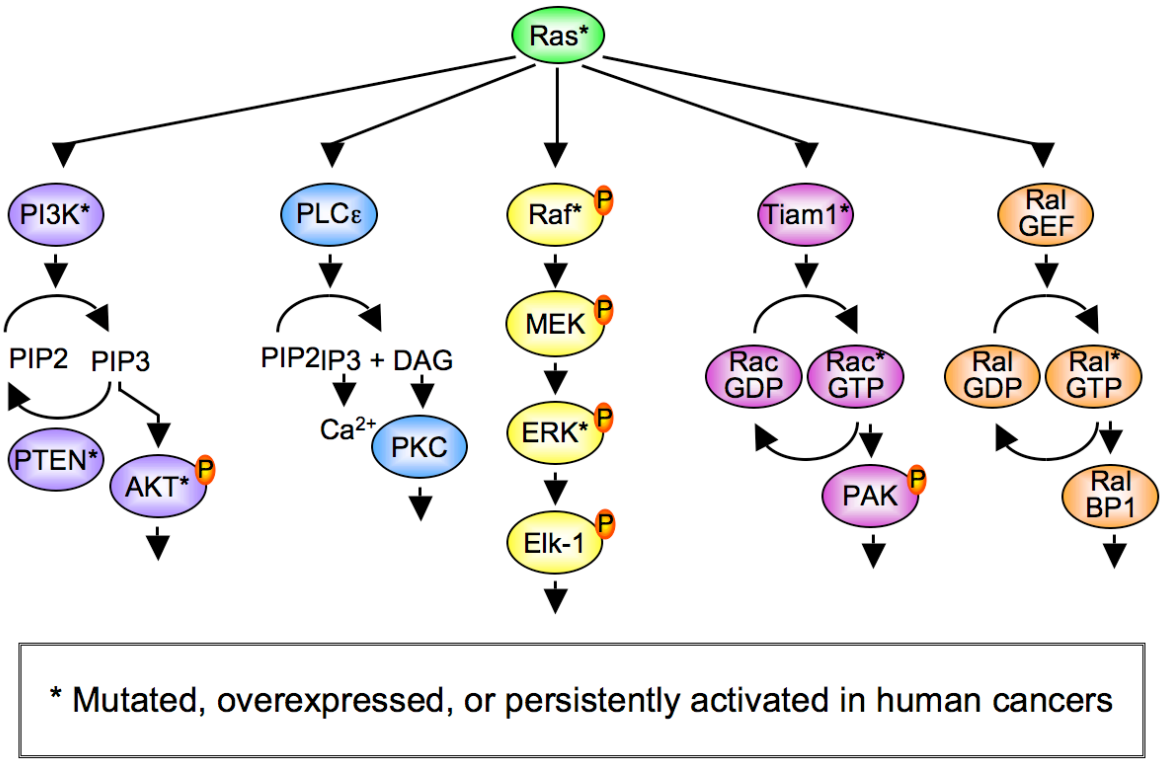


Figure 1-5. Oncogenic Ras Effector Pathways.
Currently five effector classes have been demonstrated to have necessary roles in Ras-mediated oncogenesis: PI3K, PLCε, Raf, Tiam1, and RalGEF. Among these effector pathways, several components are mutated, overexpressed, or persistently activated in human cancers (denoted by asterisks).

K-Ras mutational status is not predictive of Raf-MAPK activation, as assayed by ERK phosphorylation, in pancreatic cancer (Lim et al., 2005; Yip-Schneider et al., 1999). This suggests that Ras may utilize other effector pathways to promote pancreatic cancer.

The p110 catalytic subunits (p110α, β, δ, and γ) of class I phosphoinositide 3-kinases (PI3K) are the second best-characterized Ras effectors (Rodriguez-Viciana et al., 1994). PI3K has an important role in mediating pro-survival and proliferation functions of Ras (Cox and Der, 2003). Upon activation, PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate to form phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). PIP_3 activates the serine/threonine kinase AKT, which leads to upregulation of the transcription factor NF-κB. The PI3K pathway has been found to be hyperactive in cancer due to several mechanisms (Wong et al., 2010): loss of the dual specificity phosphatase PTEN (Yin and Shen, 2008), mutational activation of the p110α catalytic subunit (Samuels et al., 2004) and aberrant overexpression and activation of AKT (Vivanco and Sawyers, 2002). All three of these mechanisms leading to hyperactivation of the PI3K pathway have been seen in pancreatic cancer (Altomare et al., 2002; Asano et al., 2004; Schonleben et al., 2006). However, like the ERK MAPK pathway, PI3K pathway activation, as assayed by AKT phosphorylation, does not correlate with K-Ras mutational status in pancreatic cancer (Lim et al., 2005; Lim et al., 2006). Thus the role of PI3K in pancreatic cancer may be Ras-independent.

The Ral guanine nucleotide exchange factors (RalGEFs) were initially thought to play a minor role in Ras-mediated oncogenesis as evaluated in rodent fibroblast

model cell systems. Activation of Raf or its downstream targets has been well established in the induced tumorigenic transformation of these rodent fibroblast cell lines (Leevers et al., 1994; Shields et al., 2000; Stokoe et al., 1994). Dominant-negative mutants of c-Raf-1, MEK, and ERK effectively blocked Ras transformation (Cowley et al., 1994; Khosravi-Far et al., 1995; Kolch et al., 1991; Schaap et al., 1993). Furthermore, constitutively activated forms of the other main Ras effectors, PI3K or RalGEF, did not demonstrate potent transformation (Collette et al., 2004; McFall et al., 2001; Rodriguez-Viciana et al., 1997; Ulku and Der, 2003; Wolthuis et al., 1997). PI3K and RalGEF, however, can cooperate with activated Raf to induce synergistic transforming activity (Urano et al., 1996; White et al., 1996). This led to the premature conclusion that RalGEFs are not key players in Ras transformation.

However, evidence suggests that oncogenesis is not facilitated by the same mechanisms in rodents and humans (Rangarajan and Weinberg, 2003). In support of this, recent studies showed that signaling through RalGEF, and not Raf or PI3K, is necessary and sufficient for transformation in human embryonic kidney epithelial cells (HEK) (Figure 1-6) (Hamad et al., 2002). Species variation and not cell lineage (mesoderm vs. ectoderm) is thought to underlie the discrepancies in Ras-induced transformation in humans and mice because activation of RalGEF effector signaling alone was also sufficient to transform a variety of other human cells including fibroblasts (Hamad et al., 2002).

The RalGEF-Ral pathway has been further demonstrated to be critical for the tumorigenic, invasive and malignant growth of pancreatic carcinomas (Lim et al., 2005; Lim et al., 2006). The RalGEF-Ral pathway was also shown to be necessary

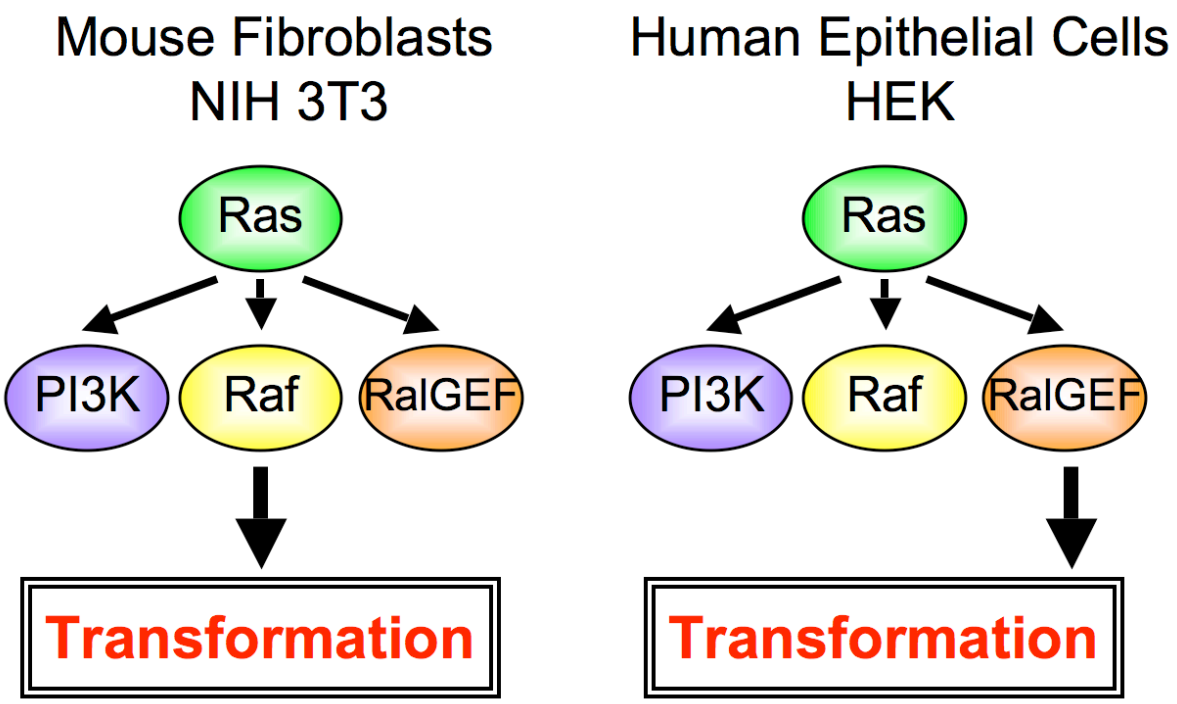


Figure 1-6. Species Variation of Ras Effector Pathways Important for Transformation. Raf and RalGEF have different roles in Ras-mediated transformation of mouse and human cells. Raf, but not PI3K or RalGEF, activation is sufficient for Ras-mediated transformation of NIH 3T3 mouse fibroblasts. However, RalGEF, but not Raf or PI3K, activation is sufficient and necessary for Ras-mediated transformation of HEK human embryonic kidney cells and a variety of other human cells including fibroblasts.

for promotion of prostate carcinoma metastasis to bone (Yin et al., 2007). Additionally, generation of mice with homozygous deletion of one RalGEF isoform (RalGDS) results in delayed onset, decreased incidence, and decreased size of carcinogen-induced skin papillomas (Gonzalez-Garcia et al., 2005). These studies suggest that RalGEF-Ral is a critical effector pathway in oncogenic Ras-induced transformation of human epithelial cells and carcinoma growth.

IV. RalGEF-Ral Signaling Pathway

A. Ras-dependent and –independent RalGEF Activation

RalGEFs are a family of guanine nucleotide exchange factors for the Ral small GTPases. Four human RalGEFs that contain a common C-terminal Ras-association (RA) domain have been identified: RalGDS, RGL, RGL2/Rlf, and RGL3 (Figure 1-7A). GTP-bound Ras relocates these RalGEFs to the plasma membrane where they promote the exchange of GDP for GTP on RalA and RalB (Kishida et al., 1997; Matsubara et al., 1999). The RalGEF pathway has also been implicated in signaling by other members of the Ras family branch of the Ras superfamily. It has been suggested by one study in *Drosophila melanogaster* and various studies in mammalian systems that the small GTPase Rap (Ras proximate) may activate RalGEFs (Mirey et al., 2003; Wolthuis et al., 1998). *In vitro,* GTP-bound Rap tightly associates with one of the RalGEFs, RalGDS (Herrmann et al., 1996). In fact, Rap has been previously shown to bind RalGDS with a higher affinity than does Ras. In reconstitution experiments with lipid vesicles, Rap was shown to stimulate RalGEF-

mediated Ral activation (Kishida et al., 1997). However, a limited number of studies have suggested that Rap does not lead to the activation of Ral in mammalian cell lines (Zwartkruis et al., 1998). Consequently, the role of Rap in RalGEF-Ral signaling has not conclusively been determined. Another potential upstream regulator of the RalGEFs are the three R-Ras proteins (Spaargaren and Bischoff, 1994). A study utilizing transient over-expression analyses with three human RalGEFs showed that mutationally activated R-Ras, TC21/R-Ras2, M-Ras/R-Ras3, Rit and Rap1, but not Rap2 or Rin, caused different levels of Ral-GTP formation in mammalian cells (Rodriguez-Viciana et al., 2004). However, whether these Ras family GTPases can activate RalGEF-Ral signaling under physiologic situations of endogenous activation and expression has not yet been determined.

An additional family of RalGEFs that do not contain a C-terminal RA domain has more recently been identified (de Bruyn et al., 2000; Rebhun et al., 2000). This family of RalGEFs is known as RalGPS (Ral GEFs with PH domain and SH3-binding motif) and contains two family members, RalGPS1A (also called RalGEF2) and RalGPS1B. These GEFs contain a characteristic N-terminal catalytic CDC25 homology domain followed by a central proline rich PxxP motif and a C-terminal pleckstrin homology (PH) domain (Figure 1-7B). PxxP motifs are known binding sites for Src homology 3 (SH3) domain containing proteins and PH domains can bind Phosphatidylinositol lipids within membranes. Unlike RA-domain containing RalGEFs that require Ras for proper membrane localization, RalGPS is regulated by its PH domain and Grb2-binding to its PxxP motif (Rebhun et al., 2000). The PH domain is thought to constitutively anchor RalGPS to the membrane. In support of

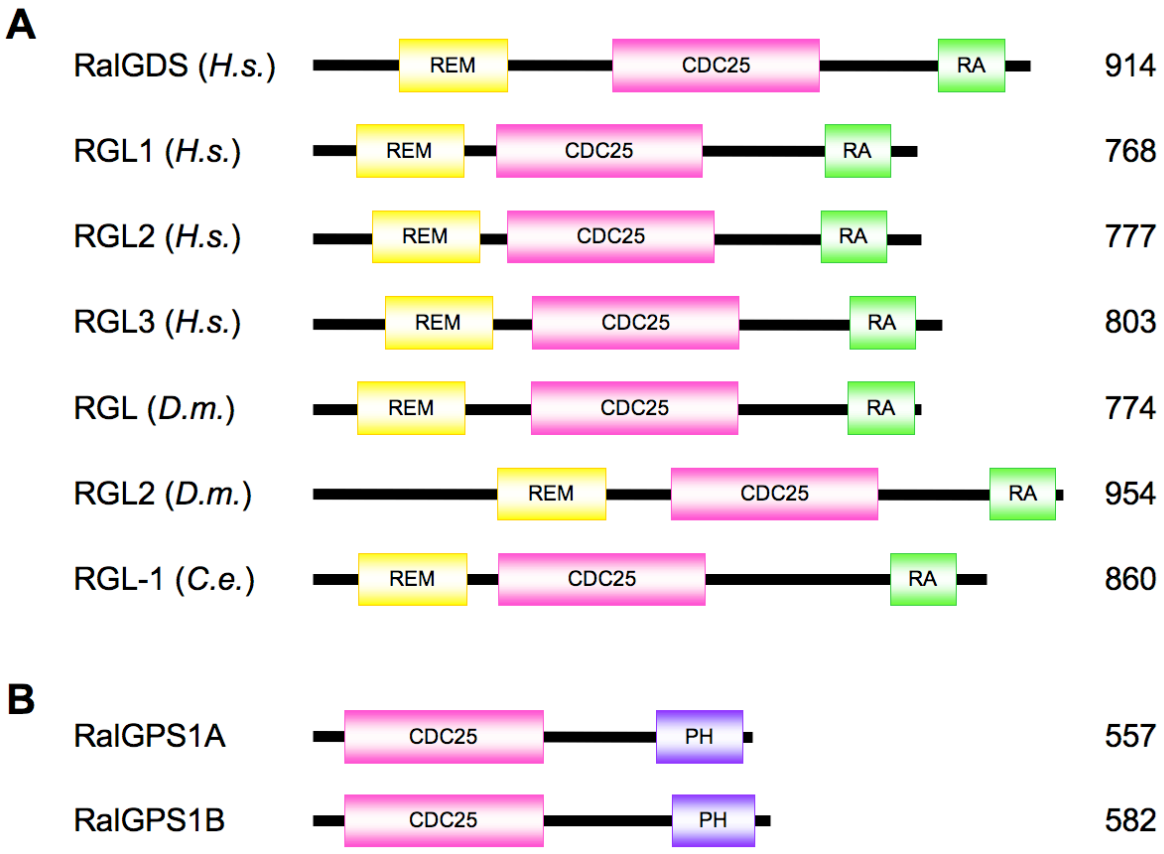


Figure 1-7. Domain Architecture of the RalGEF Family.
(A) In humans (*H.s.*), there are four isoforms of RalGEF that contain a Ras Association (RA) domain. There is one ortholog in *C. elegans* (*C.e.*) and two in *Drosophila* (*D.m.*). The RA domain-containing RalGEFs all share identical domain architecture: an N-terminal Ras Exchange Motif (REM), a central CDC25 homology (CDC25) catalytic domain, and a C-terminal Ras-GTP Association (RA) domain. The *C. elegans* ortholog, RGL-1 is best conserved with RalGDS. **(B)** An additional family of RalGEFs, RalGPS1A and RalGPS1B, lack the RA domain and are thus thought to be regulated independently of Ras activation. The domain architecture of the RalGPS family contains a catalytic CDC25 domain followed by a C-terminal pleckstrin homology domain (PH).

this, deletion of the PH domain results in increased cytoplasmic localization and decreased Ral activation (de Bruyn et al., 2000). Thus, it is hypothesized that the activity of the RalGPS family is regulated independently of Ras activation. It is currently unknown whether RalGPS activation of Ral plays a role in tumorigenesis.

B. Similarity of Sequence and Effectors of Ral Isoforms

Ral activity, like other small GTPases, is regulated by GDP/GTP switching. Although several GEFs for Ral have been identified, negative regulators, specifically RalGAPs, have remained elusive. A very recent study identified the first RalGAP complexes, RalGAP1 and RalGAP2 (Shirakawa et al., 2009). These RalGAP complexes are composed of two subunits: catalytic α1 or α2 and a common β subunit. The RalGAPs are structurally similar to the tuberous sclerosis tumor suppressor complex (TSC1/TSC2; GAP for the small GTPase Rheb). Like TSC1/TSC2, dimerization of the two RalGAP subunits (α1 or α2 with β) is required for RalGAP activity.

There are two isoforms of Ral in humans, RalA and RalB, whose genes are ubiquitously expressed, and encode proteins that are 82% identical. RalA and RalB are 100% identical in their effector domains (corresponding to residues 25-45), with the majority of the differences in the two isoforms lying within the hypervariable C-terminus involved in membrane targeting (Figure 1-8). Once activated, Ral proteins regulate many cellular processes including endocytosis, exocytosis, actin cytoskeletal dynamics, and transcription. Several Ral effectors and binding proteins have been identified including: RalBP1/RLIP76, a Cdc42- and Rac-specific

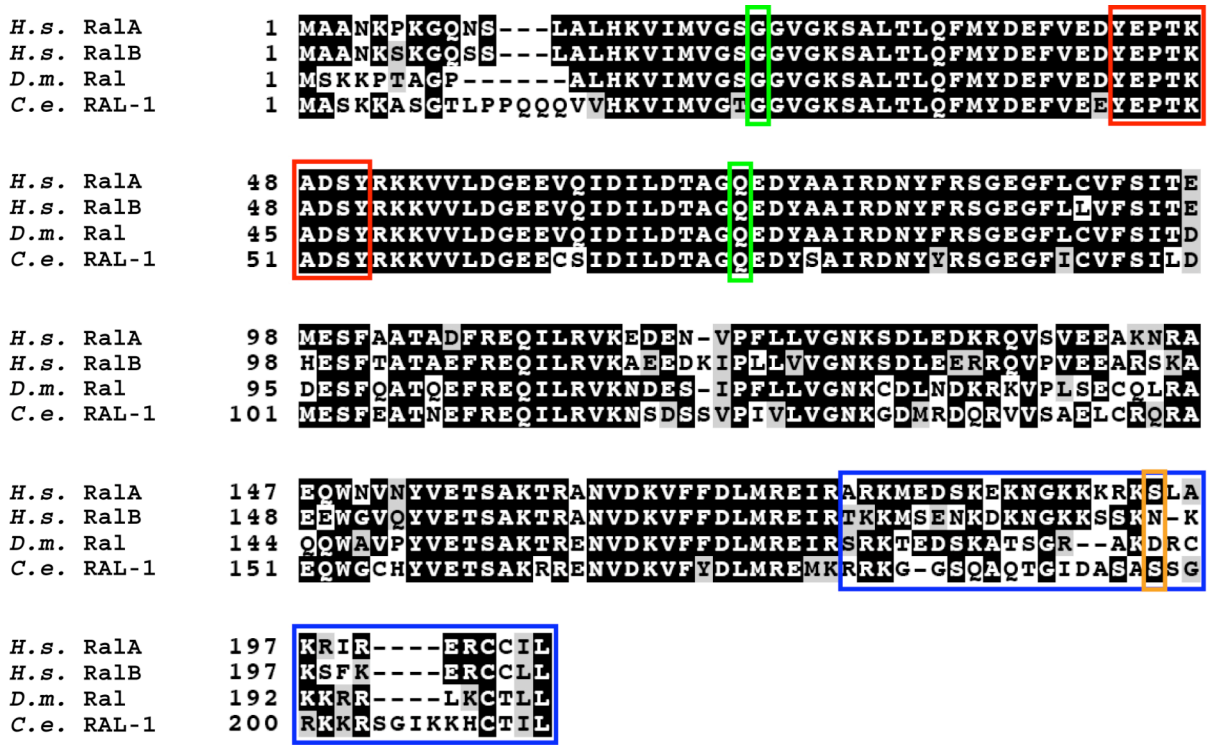


Figure 1-8. Alignment of Ral Orthologs.
ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used to align full-length protein sequences of *Homo sapiens* (*H.s.)* RalA (NCBI Accession NP_005393), *H.s.* RalB (NP_002872), *Drosophila melanogaster (D.m.)* Ral (NP_525063), and *Caenorhabditis elegans (C.e.)* RAL-1 (NP_497689). Sequences were shaded using Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Greater than 50% identical or similar residues are marked with black or grey shading, respectively. Key residues for GAP stimulation of GTP hydrolysis (boxed in green) and the core effector domain (boxed in red) are 100% conserved amongst species. The majority of differences are in the C-terminal hypervariable and CAAX prenylation signal motif region (boxed in blue). The C-terminal leucine residue suggests that RAL-1, like human Ral proteins, is C-terminally modified with a geranylgeranyl lipid moiety (Falsetti et al., 2007). Serine 194, which is an Aurora-A phosphorylation site (Wu et al., 2005), is conserved in *C.e.* RAL-1 but not *H.s.* RalB or *D.m.* Ral.

RhoGAP; the Sec5 and Exo84 subunits of the octomeric exocyst complex; the actin binding protein filamin; phospholipase D1 (PLD1); and the ZO-1 associated nucleic acid-binding protein (ZONAB) (Cantor et al., 1995; Chien et al., 2006; Frankel et al., 2005; Jullien-Flores et al., 1995; Luo et al., 1997; Moskalenko et al., 2002; Moskalenko et al., 2003; Ohta et al., 1999; Park and Weinberg, 1995). Additionally, Ral signaling can activate various transcription factors including: NF-κB, AFX (FOXO4), TCF, c-Jun, and cyclin D (Figure 1-9) (de Ruiter et al., 2000; Goi et al., 2000; Henry et al., 2000; Kops et al., 1999). The exact roles these effectors play in Ral signaling are poorly understood. Since RalGEF-Ral signaling is important in Ras-mediated transformation of human cells, understanding how RalGEF-Ral signaling is propagated may provide insight into oncogenic Ras tumorigenicity.

C. The Antagonistic Relationship of RalA and RalB

Despite their similarity in sequence and effectors, RalA and RalB appear to perform different and in some cases antagonistic functions. In one study, while RalA was required for the anchorage-independent proliferation of human tumor cells, RalB was required for tumor but not normal cell survival (Chien and White, 2003). Counter and colleagues found that RalA but not RalB was required for the anchorage-independent and tumorigenic growth of Ras-transformed human cells and pancreatic carcinoma cells, whereas RalB was necessary for invasion and metastasis (Lim et al., 2005; Lim et al., 2006). In human colorectal carcinoma cell lines, knockdown of RalA expression suppressed anchorage-independent growth, whereas knockdown of RalB expression greatly stimulated growth (Martin et al.;

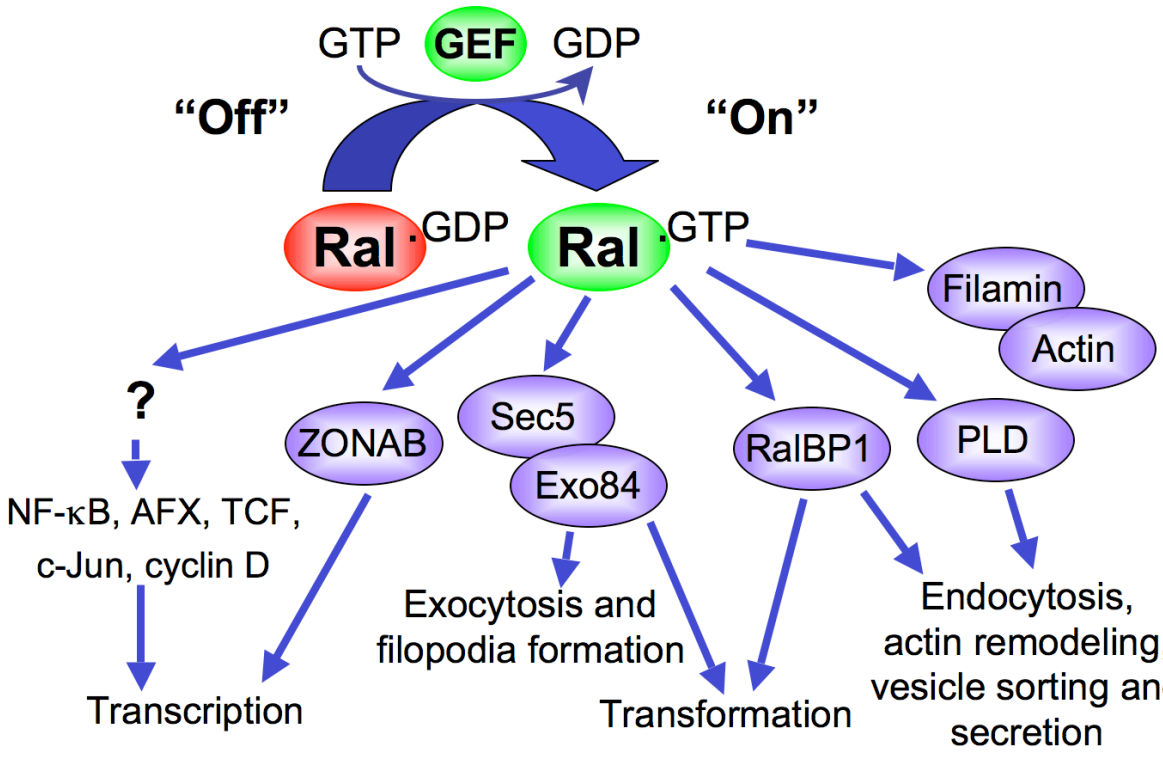


Figure 1-9. Ral Signal Transduction.
Several Ral effectors and binding proteins have been identified biochemically. Ral effectors include: RalBP1/RLIP76, a Cdc42- and Rac-specific RhoGAP; the Sec5 and Exo84 subunits of the exocyst complex; the actin binding protein filamin; phospholipase D1 (PLD1); and the Zo-1 associated nucleic acid-binding protein (ZONAB). RalGEF-Ral signaling can also lead to activation of various transcription factors including: NFκB, AFX (FOXO4), TCF, c-Jun, and cyclin D.

personal communication). Cancer cell migration appears to also be regulated antagonistically by RalA and RalB. Recently it was shown in a human bladder cancer cell line, UMUC-3, that RalA inhibits motility, whereas RalB is promigratory (Oxford et al., 2005). Taken together these studies indicate distinct roles for both RalA and RalB in cancers that additionally vary in different cancer cell types. These observations suggest that RalA and RalB collaborate to promote cell proliferation and survival in Ras-mediated oncogenesis. In support of this, activated GTP-bound forms of both RalA and RalB are significantly elevated in human pancreatic carcinoma cell lines, pancreatic tumors, and bladder tumors (Chien et al., 2006; Lim et al., 2005; Lim et al., 2006; Smith et al., 2007).

The differences in function of RalA and RalB may be due, in part, to their distinct subcellular localizations (Shipitsin and Feig, 2004). Activated forms of both RalA and RalB have been shown to localize to the plasma membrane. Additionally, RalA also localizes to recycling endosomes. It has been previously shown that RalA mutants that do not localize to recycling endosomes fail to promote basolateral secretion through the exocyst complex (Shipitsin and Feig, 2004). Furthermore, replacing the C-terminal membrane targeting sequence of RalB with the corresponding region of RalA targets RalB to recycling endosomes, and endows RalB with the ability to promote anchorage-independent growth (Lim et al., 2005). Conversely, replacing the C-terminus of RalA with the corresponding region of RalB decreased its ability to promote anchorage-independent growth. However, mislocalization of RalA did not completely abolish RalA-induced anchorage-

independent growth. This suggests that the subcellular localization of RalA and RalB does not fully account for the functional differences.

Additionally, distinct downstream effector utilization may account for the functional differences of RalA and RalB. It has been previously demonstrated that RalA has a higher affinity for exocyst binding than RalB (Shipitsin and Feig, 2004). It was also shown that active RalA but not RalB promotes exocyst-related functions in cells as measured by basolateral delivery of E-cadherin. Though RalB has a lower affinity for the exocyst complex, it has been recently shown to engage the exocyst subunit Sec5 in a manner independent of canonical exocyst function (Chien et al., 2006). The RalB-Sec5 effector complex recruits and activates an atypical IκB serine/threonine kinase family member TBK-1 (Tank binding kinase 1). Studies showed that TBK-1 is chronically activated in several cancer cell lines, and required for the growth of Ras mutant and wild type human tumor cell lines (Barbie et al., 2009; Chien et al., 2006). This Sec5-TBK-1 pathway was shown to mediate the anti-apoptotic activity of RalB. Further elucidation of the effector pathways engaged by RalA and RalB may shed light onto their differential functions in promoting cancer initiation, progression, and migration.

Furthermore, differences in location, effector utilization and subsequent function may be attributed to additional regulation of RalA, but not RalB, by phosphorylation. Recently, it was discovered that serine 194 in the C-terminus of human RalA is an Aurora-A serine/threonine mitotic kinase phosphorylation site (Figure 1-8) (Wu et al., 2005). This residue, however, is not conserved in human RalB, and thus RalB is not a substrate for Aurora-A. Studies showed that

phosphorylation of RalA by Aurora-A leads to elevated levels of activated RalA-GTP (Wu et al., 2005). RalA phosphorylation was also shown to promote relocalization of RalA from the plasma membrane to internal membranes (Lim et al., 2010). This internalization significantly enhanced the association of RalA with RalBP1 resulting in an increase in RalBP1 GAP activity. It was also found that activated Aurora-A cooperated synergistically with RalGEF-Ral to promote transformation of immortalized human cells, and that pancreatic cancer cells in culture harboring oncogenic Ras mutations depended on RalA S194 phosphorylation for transformation (Lim et al., 2010). In further support of the importance of RalA phosphorylation, it was found that the tumor suppressor protein phosphatase 2A (PP2A) normally restricts tumor progression, in part, through dephosphorylation of RalA at S194 (Sablina et al., 2007). These studies suggest that the phosphorylation status of RalA regulates its tumorigenic activity.

D. Therapeutic Targeting of Ral

The increasing evidence for RalGEF-Ral as a key mediator of Ras oncogenesis has led to efforts to identify approaches for blocking Ral function. As mentioned above, recent studies have suggested that Aurora-A inhibitors may be effective at blocking RalA-mediated oncogenesis. Aurora-A has been separately validated as a target for anti-cancer therapeutics. Aurora-A originally drew attention as a possible anti-cancer drug target because of its frequent overexpression in human cancers (Giet et al., 2005; Katayama et al., 2003; Keen and Taylor, 2004). Specifically, Aurora-A has been found overexpressed in pancreatic cancer by either

gene amplification or elevated levels of mRNA or protein (Fukushige et al., 1997; Li et al., 2003). There are currently twelve Aurora kinase inhibitors in Phase I/II clinical trials (Dar et al., 2010). Clinical tolerability has generally been good; however several studies have found neutropenia (low level of neutrophils; a type of white blood cell) to be the primary dose-limiting toxicity (Cheung et al., 2009; Dar et al., 2010). Additionally, Aurora kinase inhibitors have been found to induce polyploidy in cell culture (Ditchfield et al., 2003). Whether effective anti-tumor activity can be achieved with doses of Aurora kinase inhibitors that do not result in adverse long-term clinical effects is unknown. Also, whether Aurora-A inhibitors will be effective at treating oncogenic Ras-driven patient tumors remains unclear.

Like Ras, Ral GTPases require proper subcellular localization for biological activity (Lim et al., 2005). Both RalA and RalB terminate with a C-terminal CAAX motif. However unlike Ras, which is farnesylated, geranylgeranyltransferase-I (GGTase-I) catalyzes the addition of a 20-carbon geranylgeranyl isoprenoid lipid to the cysteine residue of the Ral GTPase CAAX motif (Falsetti et al., 2007; Kinsella et al., 1991). Thus, geranylgeranyltransferase-I inhibitors (GGTIs) may block the oncogenic activity of RalA and RalB. In mouse models of cancer, GGTIs have been shown to inhibit tumor growth and induce tumor regression (Sun et al., 1999; Sun et al., 2003). The specific GGTase-I substrates that are important for the antitumor activity of GGTIs are not known. A recent study found that inhibition of RalA partly mediates the effects of GGTIs on anchorage-independent growth, whereas inhibition of RalB mediates the effects of GGTIs on apoptosis (Falsetti et al., 2007). This study suggests that RalA and RalB are key targets that account for the

antineoplastic activities of GGTIs. However, many proteins are substrates for GGTase-I, and further studies need to be performed to identify the critical targets for GGTIs antitumor properties (Reid et al., 2004). Further understanding and characterization of Ral pathway upstream regulation and downstream effectors important in tumorigenesis will be critical for future development of effective therapeutics.

E. RalGEF-Ral Conservation Across Species

Invertebrate genetic model systems have played a central role in the dissection of the Ras signal transduction cascades. In particular, genetic studies in the model system *C. elegans* were critical in identifying many of the Ras-Raf pathway components. The Ras-Raf signaling pathway is highly conserved among metazoans. For example, LET-60/Ras shares 73-77% amino acid sequence identity with human H-, N-, and K-Ras proteins, and residues critical for effector binding and activation (residues 32-40) are 100% conserved (Figure 1-2). SMART database (http://smart.embl-heidelberg.de/) analyses for *C. elegans* proteins with Ras Binding Domain (RBD) or RA domain homologies identifies three other Ras effectors with conserved orthologs in *C. elegans*: PI3K (AGE-1), PLCε (PLC-1) and RalGEF (RGL-1) (Figure 1-10). To date, no role for any of these candidate LET-60 effectors has been evaluated in vulval development. In light of the growing importance of the RalGEF-Ral pathway in K-Ras mutant pancreatic cancers, I have chosen to focus my dissertation research on genetically dissecting the role of RGL-1 in LET-60 regulation of vulva development.

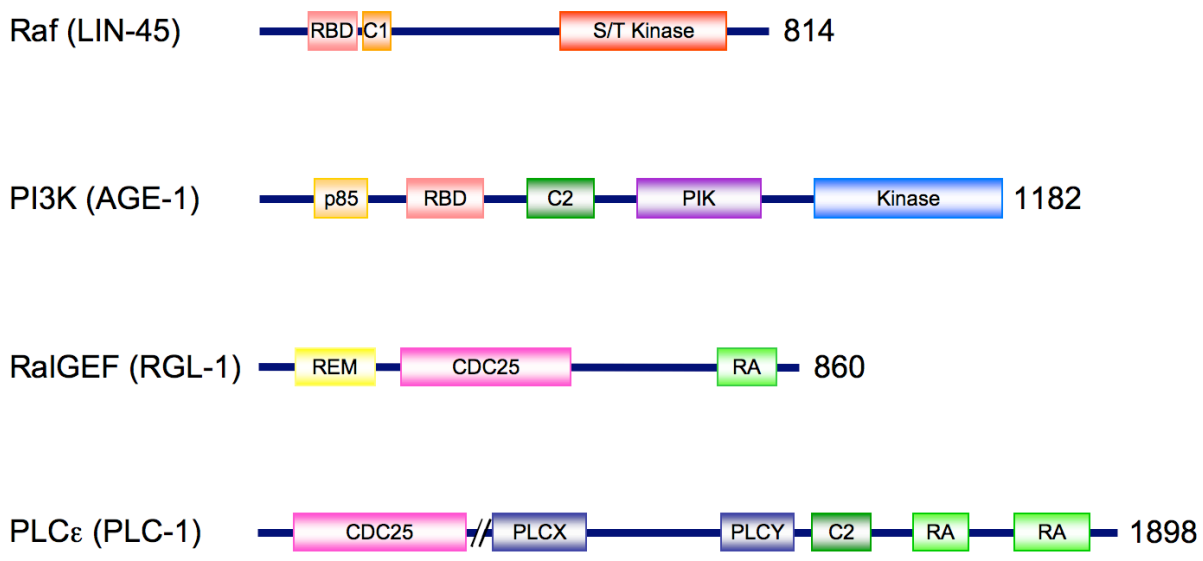


Figure 1-10. Conserved Ras Effector Orthologs in *C. elegans*.
SMART database (http://smart.embl-heidelberg.de/) analysis identified four *C. elegans* proteins containing either a Ras Binding Domain (RBD) or Ras Association Domain (RA): LIN-45 (Raf), AGE-1 (PI3K), RGL-1 (RalGEF), PLC-1 (PLCε). Additional domains are as follows: Protein Kinase C Conserved Region 1 (C1), Serine/ Threonine Kinase (S/T Kinase), PI3K Family p85-Binding Domain (p85), Protein Kinase C Conserved Region 2 (C2), PI3K Family Accessory Domain (PIK), PI3K Catalytic Domain (Kinase), Ras Exchange Motif (REM), CDC25 Homology Catalytic Domain (CDC25), Phospholipase C Catalytic Domain X (PLCX), and Phospholipase C Catalytic Domain Y (PLCY).

Genes encoding RalGEF and Ral orthologs, like Ras, are also highly conserved across species. There are single RalGEF (RGL-1) and Ral (RAL-1) orthologs in *C. elegans*. RGL-1 contains the expected RalGEF domain architecture: a Ras Exchange Motif (REM) domain, followed by a CDC25 homology RasGEF domain, and a Ras Association (RA) domain (Figure 1-7A). RGL-1 is most similar to human RalGDS, with the GEF domain being the most highly conserved. Typical for a small GTPase, RAL-1 consists of a GTPase domain that shares remarkable identity with its human orthologs, followed by the relatively short C-terminal hypervariable and CAAX regions (Figure 1-8). RAL-1 is ~65% identical to human RalA and RalB, with the majority of the divergence in the C-terminal hypervariable region. The effector binding regions of the Ral GTPases share very high identity, with the core G2 box containing key residues for GEF and GAP regulation and effector binding (RAL-1 residues 39-59) being 95% conserved among *H. sapiens, C. elegans*, and *D. melanogaster* homologs.

C. elegans is an excellent model system for mechanistic analysis of signal transduction. The short replication time and defined genome of *C. elegans* allows for rapid screening through downstream candidates and possible identification of pathway components. *C. elegans* studies may also provide insight into the role of proteins *in vivo*. The strong sequence conservation of components of the Ras-RalGEF-Ral signaling module across species suggests that the biochemical circuitry of this Ras effector pathway is evolutionarily conserved. Thus, studies in *C.* elegans could shed light on the normal *in vivo* functions of RalA and RalB in humans. The

conservation of pathways combined with the powerful genetic toolkit of *C. elegans* makes it an excellent model for studying Ral.

V. *C. elegans* Vulval Development

A. Vulval Competence Group

One of the best-studied developmental features of *C. elegans* is the formation of its egg-laying organ, the vulva (Sternberg, 2005). The *C. elegans* vulva develops during the larval L3 post-embryonic stage from ventral epithelial vulval precursor cells. In the adult hermaphrodite, this ventrally situated epithelial aperture provides a connection between the uterus and the external environment and is necessary for egg-laying. Development of the *C. elegans* vulva is a paradigm for genetic analysis of tissue patterning. Use of this model system has resulted in broad insights into developmental biology and signal transduction.

During the first larval stage (L1), the posterior daughters of the ventral neuro-ectoblasts (Pn cells) divide giving rise to the Pn.p cells (P1.p-P12.p) (Sulston and Horvitz, 1977). At the end of the L1 stage, P1.p, P2.p and P9.p-P12.p fuse with the epidermal syncytium (hyp 7). Expression of homeotic selector (Hox) genes, particularly the HOM-C gene *lin-39*, specifies the six remaining Pn.p cells to make up the vulval precursor competence group (P3.p-P8.p) (Clark et al., 1993; Salser et al., 1993). Lack of *lin-39* expression, as analyzed in *lin-39* null mutants, results in the presumptive vulval precursor cells (VPCs) fusing with the hyp-7 epidermis.

The six VPCs (P3.p-P8.p) are competent to respond to intercellular signals and generate the vulva. The VPCs are equivalent in developmental potential and can assume any of three alternative fates (1°, central vulva; 2°, lateral vulva; or 3°, non-vulval) (Sternberg and Horvitz, 1986; Sulston and White, 1980). Mutational activation of signaling pathway components that specify VPC fates (e.g., LET-23/EGFR or LIN-12/Notch) result in only P3.p-P8.p adopting vulval fates (Ferguson et al., 1987; Greenwald et al., 1983; Sternberg, 1988). Additionally, ablation of P3.p-P8.p does not result in vulval progeny from P2.p or P9.p (Sulston and White, 1980). These studies suggest that only P3.p-P8.p form the vulval equivalence group (Sulston and White, 1980).

B. Vulval Precursor Patterning

Patterning of the VPCs to generate the vulva is coordinated by the inductive cell-signaling activity of a gonadal cell called the anchor cell (AC). Signaling from the AC is necessary for vulval development as ablation of the AC before the L3 stage blocks vulval development, resulting in all VPCs adopting the 3° non-vulval fate (Kimble, 1981). The AC induces VPCs to assume a highly reproducible 3°-3°-2°-1°-2°-3° pattern of fates (Figure 1-11A). The inductive signal from the AC is a secreted EGF-like peptide growth factor, LIN-3. In a wild-type hermaphrodite, LIN-3/EGF secreted from the AC binds to LET-23/EGFR and signals through the cascade of LET-60/Ras, LIN-45/Raf, MEK-2/MEK, MPK-1/ERK and the LIN-1/ETS and LIN-31/HNF transcription factors to specify the 1° cell fate in the cell closest to the AC, P6.p (Sundaram, 2006). The neighboring VPCs, P5.p and P7.p, adopt the

2° cell fate as a result of Notch-type (LIN-12) transmembrane receptor activation. The remaining VPCs (P3.p, P4.p, and P8.p) do not receive the inductive signal and thus adopt the non-vulval 3° cell fate, and generate nonspecific hypodermal cells (Hill and Sternberg, 1992; Moghal and Sternberg, 2003).

Since the EGFR-Ras-Raf MAPK pathway is necessary for vulval development, the *C. elegans* vulva is an ideal system for detailed analysis of Ras signaling. Loss-of-function mutations in the Ras-Raf pathway result in a vulvaless (Vul) phenotype because P5.p, P6.p, and P7.p are uninduced and adopt the non-vulval 3° cell fate (Figure 1-11C). Despite being Vul, these hermaphrodites have fertile eggs. Thus, with no aperture for egg-laying, the eggs hatch internally. Conversely, gain-of-function mutations in the Ras-Raf pathway result in a hyper-induced, multivulva (Muv) phenotype because more than three VPCs adopt 1° or 2° cell fates (Figure 1-11D) (Beitel et al., 1990; Han et al., 1990). Muv hermaphrodites generally have a single functional vulva and additional ventral protrusions (pseudovulva). Owing to these phenotypes, vulval differentiation provides a sensitive and easily scored readout of Ras signaling levels. As a result, suppressor and enhancer analyses of the *let-60(n1046*gf*)* (activated Ras) strain have identified a large number of positive and negative regulators of this pathway conserved in mammals (Moghal and Sternberg, 2003).

C. Current Models of Vulval Development

Throughout the years, genetic and cell ablation experiments have led to competing models of inductive signaling in the VPCs. The “morphogen gradient”

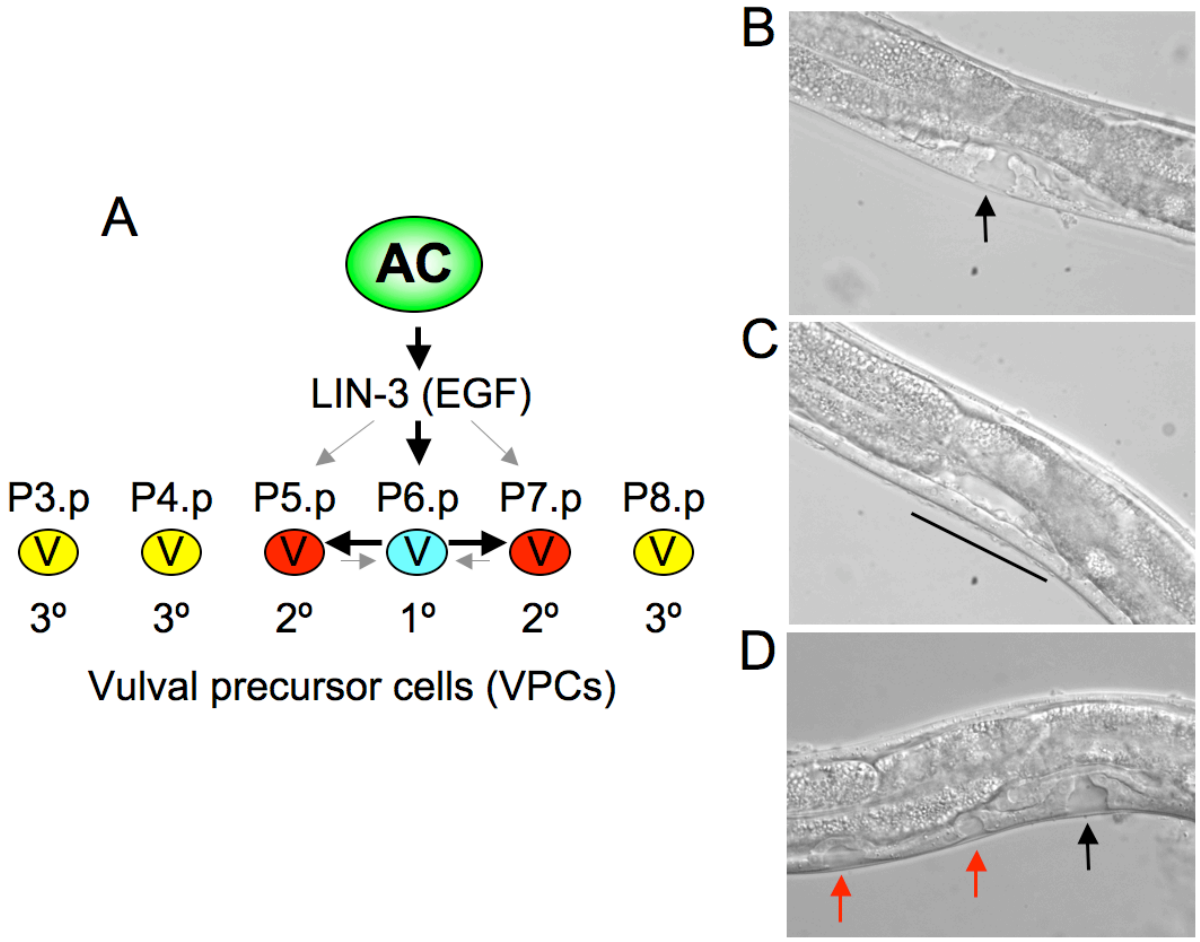


Figure 1-11. *C. elegans* Vulval Development.
(A) Vulval precursor cells (VPCs; represented as V) are multipotent ventral hypodermal cells that generate the vulva during larval stage 3 and later stages. Anchor cell (AC)-dependent EGF signaling induces the EGF-EGFR-Ras-Raf-MEK-ERK MAPK pathway to induce the 1° cell fate in the P6.p. Lateral signal from the presumptive 1° cell activates Notch in P5.p and P7.p to induce 2° fate. The remaining VPCs (P3.p, P4.p, and P8.p) do not receive the inductive signal and adopt the non-vulval 3° cell fate. 1° and 2° descendents form the vulva, and 3° descendents generate non-specific hypodermal cells (non-vulval). **(B-D)** Differential interference contrast (DIC) micrographs of animals as late L4 larvae. Anterior is left and ventral is down. The Ras-Raf MAPK pathway is necessary for vulval development. **(B)** The wild-type vulva is made up of 2°-1°-2° vulval tissue (black arrow). **(C)** Loss-of-function mutations in the Ras pathway result in an uninduced phenotype because P5.p, P6.p, and P7.p adopt the non-vulval 3° cell fate (bar). **(D)** Gain-of-function mutations in the Ras pathway result in a hyperinduced phenotype because more than three VPCs adopt 1° or 2° cell fates (red arrows indicate pseudovulvae).

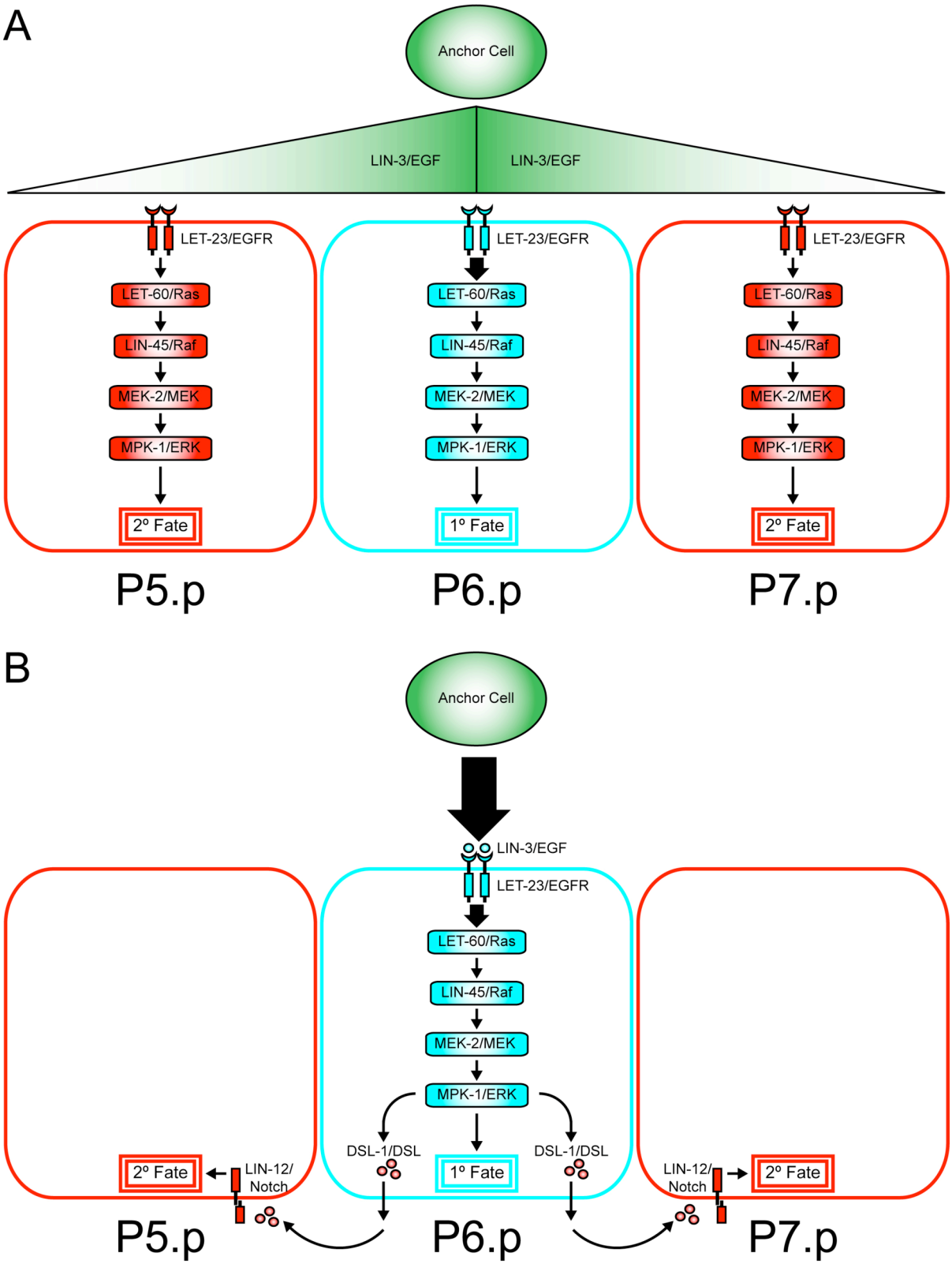
model proposes that the LIN-3/EGF inductive signal forms a concentration gradient from the anchor cell to differentially pattern the VPCs. In this model, the closest Pn.p (the P6.p) receives the highest level of inductive signal resulting in the 1° cell fate, and the neighboring Pn.ps (P5.p and P7.p) receive lower levels of the inductive signal specifying the 2° cell fate (Figure 1-12A) (Sternberg and Horvitz, 1986). This model is supported by a LIN-3/EGF heat-shock study in which varying the duration and temperature of heat-shock resulted in different doses of LIN-3/EGF. In this study, high levels of LIN-3/EGF resulted in 1° fate, intermediate levels resulted in 2° fate, and low levels resulted in 3° fate (Katz et al., 1995). Also in recent support of this model, an ERK-responsive 1° fate reporter (*egl-17::cfp)* was found to be expressed in an AC-centered gradient early in vulval induction. This reporter was expressed at high levels in the presumptive 1° VPC, with transient low levels in presumptive 2° VPCs (Yoo et al., 2004).

In contrast, the “sequential induction” model proposes that the inductive signal, LIN-3/EGF, only activates LET-23/EGFR-LET-60/Ras in the P6.p, which in turn induces the P5.p and P7.p to adopt the 2° cell fate (Figure 1-12B). The presumptive 1° cell induce its neighbors to assume the 2° fate by stimulating the expression of ligands (Delta/Serrate/LAG-2 family; DSL) for LIN-12/Notch (Chen and Greenwald, 2004). This model is supported by genetic mosaic analysis, which showed that LET-23/EGFR is necessary for normal 1° but not 2° fate induction (Koga and Ohshima, 1995; Simske and Kim, 1995). Additionally, LIN-12/Notch receptor is necessary and sufficient for 2° fate induction (Greenwald et al., 1983).

Cross talk between the LET-60/Ras and LIN-12/Notch pathways is fundamental for proper specification of the VPCs (Figure1-12C). After induction, the presumptive 1° cell, P6.p, enacts programs that down-regulate LIN-12/Notch protein through increased endocytosis (Shaye and Greenwald, 2002). Thus, LET-60/Ras-mediated inductive signaling in the P6.p has three functions. First, it specifies the 1° cell fate. Second, it causes Notch ligand production to promote 2° fate in the neighboring cells. Third, it antagonizes the pro-2° signal in the presumptive 1° cell. Conversely the LIN-12/Notch lateral signal that promotes 2° fate is also able to inhibit VPCs (P5.p and P7.p) from assuming 1° fate (Sternberg, 1988). The low levels of LIN-3/EGF–mediated inductive signal through the LET-60/Ras-LIN-45/Raf pathway are quenched in presumptive 2° cells by LIN-12/Notch transcription of negative regulators of the EGFR-ERK MAPK pathway (e.g., *lst* genes, *lip-1*/ERK phosphatase) (Berset et al., 2001; Berset et al., 2005; Yoo et al., 2004; Yoo and Greenwald, 2005). Thus, EGFR-Ras-Raf pro-1° and Notch pro-2° signals are mutually antagonistic, and while multiple studies suggest that the LIN-3/EGF inductive signal received by the P5.p and P7.p contributes to the final patterning, the mechanism of this pro-2° EGF activity is unknown.

VI. Notch Signaling

The Notch signaling pathway is also conserved across species. There are two isoforms of Notch in *C. elegans* (LIN-12 and GLP-1), one in *D. melanogaster* (Notch), and four in *H. sapiens* (Notch 1-4). Notch is a single-pass transmembrane receptor (Wharton et al., 1985) that regulates differentiation, proliferation, and


A
Anchor Cell
LIN-3/EGF
LIN-3/EGF
LET-23/EGFR
LET-60/Ras
LIN-45/Raf
MEK-2/MEK
MPK-1/ERK
2º Fate
LET-23/EGFR
LET-60/Ras
LIN-45/Raf
MEK-2/MEK
MPK-1/ERK
1º Fate
LET-23/EGFR
LET-60/Ras
LIN-45/Raf
MEK-2/MEK
MPK-1/ERK
2º Fate
P5.p
P6.p
P7.p
B
Anchor Cell
LIN-3/EGF
LET-23/EGFR
LET-60/Ras
LIN-45/Raf
MEK-2/MEK
MPK-1/ERK
DSL-1/DSL
DSL-1/DSL
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P5.p
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P7.p

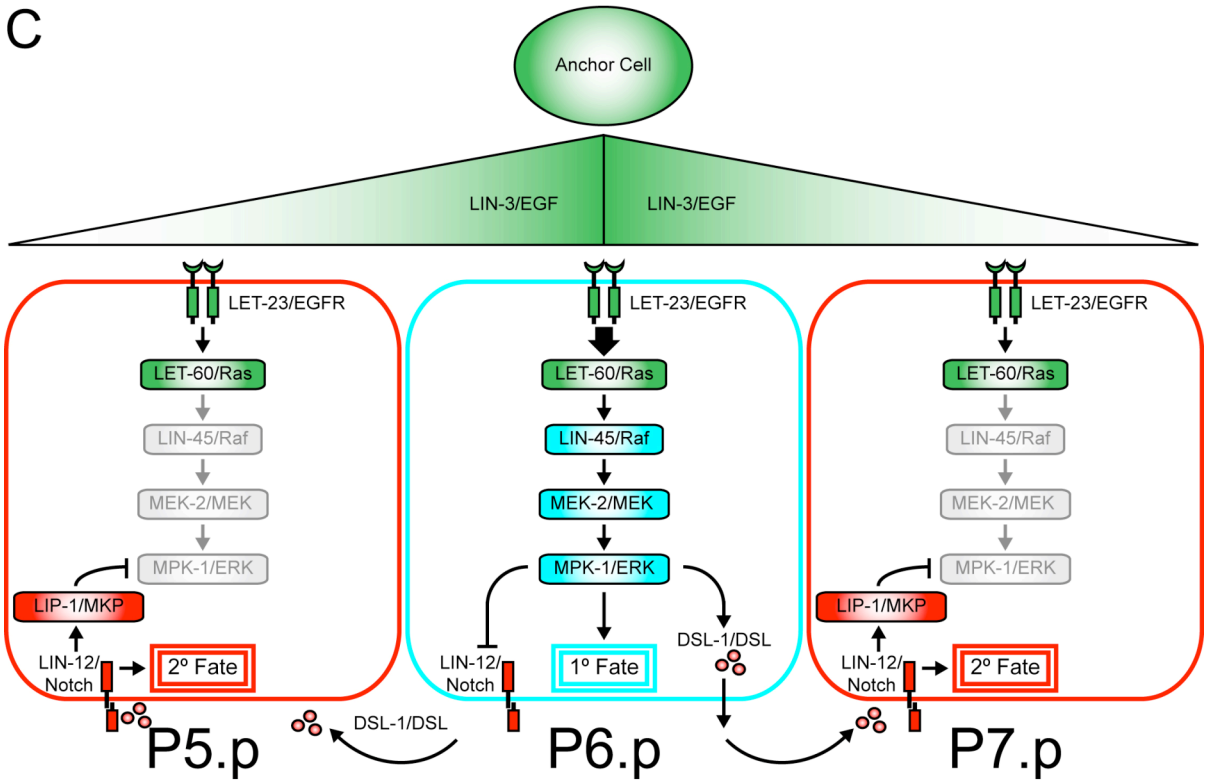


Figure 1-12. Competing Models for Vulval Fate Patterning.
Signal promoting both fates is shown in green, pro-1° signal in blue, pro-2° signal in red, and quenched signals in gray. **(A)** The "morphogen gradient model" posits that the VPCs are differentially patterned by an anchor cell secreted LIN-3/EGF concentration gradient. High levels of LIN-3/EGF signal induce the 1° cell fate (P6.p), whereas lower levels specify the 2° cell fate. Both fates are therefore specified through activation of the EGFR-Ras-Raf-MAPK pathway. **(B)** The "sequential induction model" posits that anchor cell secretion of the inductive LIN-3/EGF signal activates only the EGFR-Ras-Raf-MAPK pathway to induce the proximal VPC, P6.p, to adopt the 1° cell fate. Subsequently, this cell laterally induces the neighboring VPCs, P5.p and P7.p, via the LIN-12/Notch receptor to adopt the 2° cell fate. **(C)** Recently aspects from the "morphogen gradient" and "sequential induction" models have been combined into a "quenching model." This model proposes that cross-talk between the LET-60/Ras and LIN-12/Notch pathways is necessary for proper VPC patterning. Presumptive 1° cells enact programs to minimize conflicting pro-2° signals (e.g., LIN-12/Notch endocytosis). Conversely, LIN-12/Notch transcribes negative regulators of the Raf-MEK-ERK pathway to minimize conflicting pro-1° signals (e.g., LIP-1/MAPK phosphatase).

apoptosis (Artavanis-Tsakonas et al., 1999; Osborne and Miele, 1999). Most of the Notch ligands (DSL family) are transmembrane proteins (Wharton et al., 1985; Yochem and Greenwald, 1989). However, a class of secreted Notch ligands has also been characterized in *C. elegans* (e.g. DSL-1) (Chen and Greenwald, 2004). Upon ligand binding, Notch is cleaved by a presenilin-1-dependent γ-secretase activity (Struhl and Adachi, 1998). This proteolysis releases a C-terminal fragment of Notch (Notch intracellular domain; NICD) that translocates to the nucleus and promotes transcription (Figure 1-13) (Struhl et al., 1993).

Several mammalian studies have suggested that Notch and Ras can cooperate to promote oncogenesis. Ras and two of its effector pathways, Raf and PI3K, were found to be required for anchorage-independent growth of mouse-derived Notch-4 tumor cell lines (Fitzgerald et al., 2000). In immortalized HEK cells, constitutively active Ras was found to upregulate Delta-1 (a Notch ligand) and presenilin-1 (a Notch processing protein), resulting in an increase in the levels of 'active' Notch-1 (NICD) (Weijzen et al., 2002). Also, down-regulation of Notch-1 expression suppressed Ras-induced anchorage-independent growth. Furthermore, expression of a Notch antagonist, Deltex, inhibits H-Ras induced mouse mammary tumors (Kiaris et al., 2004).

Additionally, studies have suggested that Notch functions as an oncogene in pancreatic cancer. In pancreatic carcinoma cell lines, Notch-1-directed RNAi suppressed cell growth and invasion, whereas expression of an active form of Notch (NICD) enhanced cell growth and invasion (Wang et al., 2006a; Wang et al., 2006b; Wang et al., 2006c). Notch pathway components and target genes are also

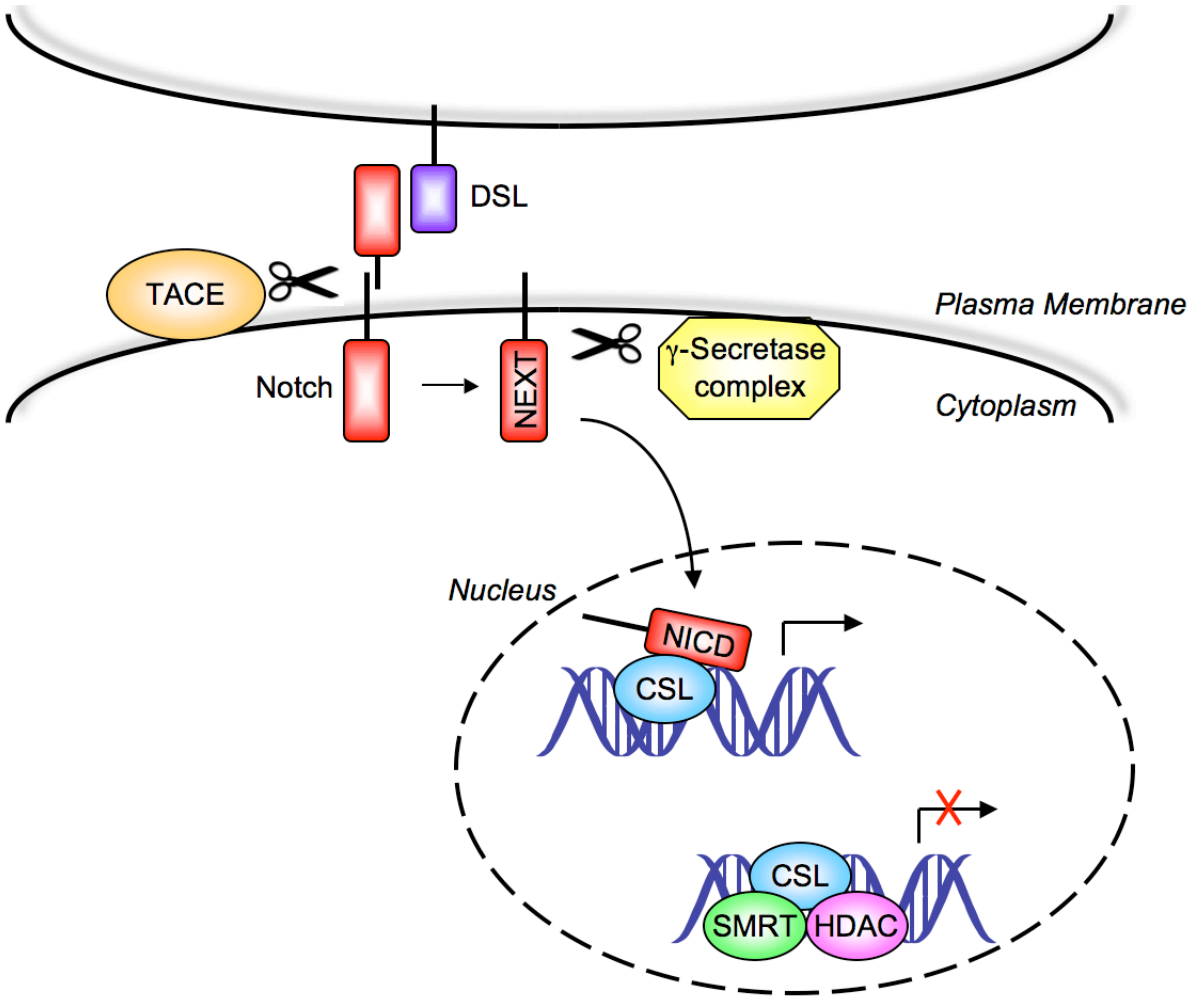


Figure 1-13. Notch Signaling.
Interaction of Notch receptors to Notch ligands (Delta/Serrate/Lag-2 family; DSL) between bordering cells triggers proteolytic cleavage of the Notch receptor. First, TNF-α-converting enzyme (TACE) mediates extracellular cleavage that generates a membrane-retained C-terminal fragment (NEXT; Notch extracellular truncated). Then, the NEXT fragment is a substrate for cleavage by the γ-secretase complex. This cleavage results in release of the Notch intracellular domain (NICD). NICD translocates to the nucleus where it associates with CSL (CBF1, suppressor of hairless). The association of NICD with CSL displaces corepressor proteins (SMRT and HDAC) leading to transcription of Notch target genes. SMRT: Silencing mediator for retinoid and thyroid hormone; HDAC: Histone deacetylase

overexpressed in pancreatic cancer (Hingorani et al., 2003; Miyamoto et al., 2003). Whether oncogenic Ras and Notch cooperate to promote pancreatic cancer is unknown. Also, the direct mechanisms by which these two pathways cooperate are unclear. Studies in *C. elegans* may elucidate the complex interplay between the Ras and Notch signaling pathways.

In summary, despite the strong evidence that aberrant Ras promotes tumor formation, the role of critical Ras downstream effectors, specifically RalGEF-Ral, in Ras-mediated oncogenesis remains poorly understood. Delineating the regulation and role of the RalGEF-Ral pathway normal function in the developing organism may aid in elucidating the mechanisms by which it is hijacked to promote cancer. In chapter two, I explore the role of the RalGEF-Ral pathway in Ras-mediated *C. elegans* vulval induction. During vulval patterning, LET-60/Ras signals through LIN-45/Raf in P.6p to antagonize LIN-12/Notch and promote the 1° fate. However, we found that in P5.p and P7.p, Ras effector utilization is switched to RalGEF-Ral, which cooperates with LIN-12/Notch to promote the 2° fate. Thus, these studies establish Ras effector switching during normal development as a mechanism by which Ras can signal for distinct cellular outcomes.

CHAPTER 2: RAS EFFECTOR SWITCHING PROMOTES DIVERGENT CELL FATES IN *C. ELEGANS* VULVAL PATTERNING[1]

[1] Authors: Tanya P. Zand, David J. Reiner, and Channing J. Der. All figures, except for 2-8E, 2-9 and table 2-1 generated by David J. Reiner, represent the work of Tanya P. Zand.

I. Abstract

The *C. elegans* vulva is patterned by epidermal growth factor (EGF) activation of Ras to control 1° fate induction and consequent Notch 2° fate induction. Furthermore a spatial EGF gradient, in addition to inducing 1° fate, directly contributes to 2° fate via an unknown pathway. We find that in addition to its canonical effector, Raf, vulval Ras utilizes an exchange factor for the Ral small GTPase (RalGEF), such that Ras-RalGEF-Ral antagonizes Ras-Raf pro-1° fate activity. Consistent with its restricted expression pattern, Ral contributes to EGF and Notch pro-2° activities. Thus, we have delineated a Ras effector-switching mechanism whereby position within the morphogen gradient dictates that Ras effector usage is switched to RalGEF from Raf to promote 2° instead of 1° fate. Our observations define the utility of Ras effector switching during normal development, and may provide one mechanism for cell and cancer type differences in effector dependency and activation.

II. Introduction

An emerging complexity of mammalian Ras signal transduction is the assortment of catalytically diverse effectors that may facilitate the elaborate biology of Ras in normal and neoplastic cells. The precise role that each effector serves, dynamic regulation of effector utilization, and interplay between effector networks are issues that remain poorly understood. Analysis of *C. elegans* vulva development has provided key insights into Ras signaling components and concepts conserved in mammalian cells. The vulval precursor cells (VPCs) are a developmental

equivalence group of six ventral epithelial cells (P3.p-P8.p) (Sternberg, 2005) (Figure 2-1A). The nearby anchor cell (AC) induces VPCs to assume a highly reproducible 3°-3°-2°-1°-2°-3° pattern of fates. The AC-proximal VPC is induced to assume the 1° fate, flanking VPCs assume the 2° fate, and distal uninduced VPCs assume the non-vulval 3° fate.

Along with studies in other systems, analyses of the pro-1° AC inductive signal were instrumental in delineating the first signal transduction pathway connecting the cell surface to the nucleus (Egan and Weinberg, 1993). The AC secretes LIN-3/EGF (epidermal growth factor), which promotes LET-60/Ras activation. GTP-bound LET-60/Ras then activates the LIN-45/Raf-MEK-ERK mitogen-activated protein kinase (MAPK) signaling cascade to regulate the LIN-1 and LIN-31 transcription factors, thereby inducing 1° fate (Sundaram, 2006) (Figure 2-1B). Analogously to human cancers, mutational activation of LET-60/Ras promotes ERK activation, leading to excess vulval induction (Figures 2-1C and 2-1D), while loss of pathway components results in vulval absence. All constituents of this signaling pathway, particularly LET-60/Ras, are strongly conserved. This degree of conservation argued, prematurely, that our understanding of Ras effector signaling was complete. However, subsequent mammalian cell studies characterized additional Ras effectors, with now at least 10 distinct functional classes identified (Repasky et al., 2004). With many effectors expressed ubiquitously, an unresolved issue is how Ras effector utilization is orchestrated to facilitate the complex biological outputs of Ras.

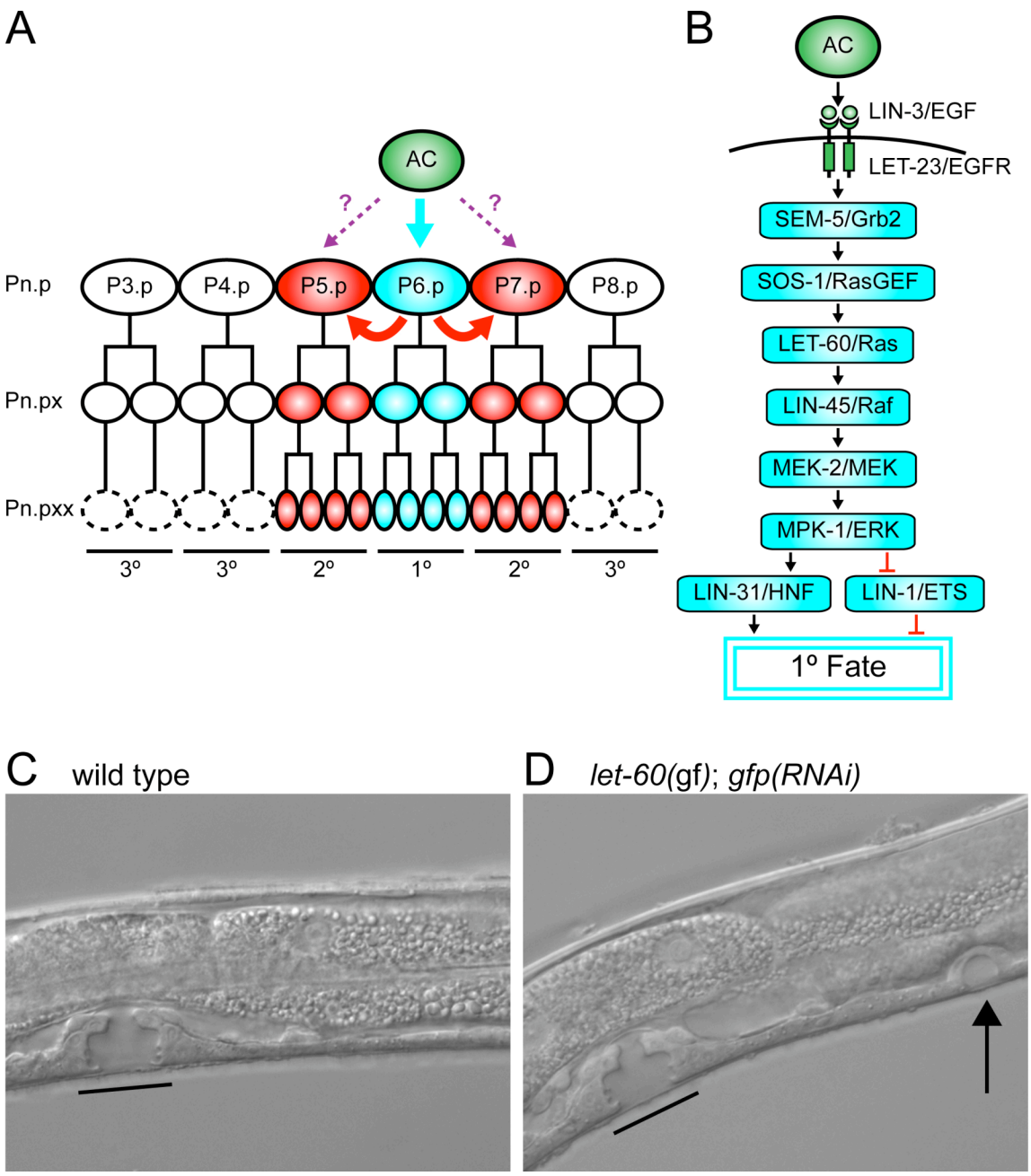


Figure 2-1. An Overview of VPC Patterning
(A) A graded EGF signal from the anchor cell (AC) induces vulval fates. High EGF levels (blue arrow) activate the EGFR-Ras-Raf pathway in P6.p to induce 1° fate. Lateral signal (red arrows) from the presumptive 1° cell activates LIN-12/Notch in P5.p and P7.p to induce 2° fate. 1° and 2° descendents form the vulva; 3° VPCs are non-vulval. Low EGF levels (purple arrows) may help pattern P5.p and P7.p. We used the transcriptional reporter $P_{egl\text{-}17}$*::cfp* (blue cells) as a marker for 1° fate (Yoo et al., 2004). **(B)** The EGF signal transduction pathway specifies 1° cell fate. **(C and D)** DIC micrographs of **(C)** wild type and **(D)** *let-60(n1046*gf*); gfp(RNAi)* animals as late L4 larvae. Bars indicate the wild-type 2°-1°-2° vulval tissue, and the arrow indicates a pseudovulva. Anterior is left and ventral down.

Recent analyses have implicated the guanine nucleotide exchange factor for the Ral GTPase (RalGEF) as an effector of importance comparable to Raf in Ras-dependent human oncogenesis (Chien and White, 2003; Hamad et al., 2002). Like Ras, Ral functions as a GDP/GTP-regulated switch. Since RalGEF and Ral are conserved in *C. elegans*, EGF activation of Ras could involve the RalGEF-Ral pathway in regulation of vulval cell fate.

Two competing models have been proposed to illustrate the mechanisms of vulval fate patterning. The original "morphogen gradient model" posits that a LIN-3/EGF, AC-maximal concentration gradient differentially patterns VPCs dictated by proximity to the AC (Katz et al., 1995; Katz et al., 1996; Sternberg and Horvitz, 1986, 1989). This model posits that while strong EGF signal induces 1° fate, diminished EGF signal directly promotes 2° fate for more distal VPCs. Appropriately, an ERK-responsive 1° fate reporter was highly expressed in the presumptive 1° VPC, with transient low expression in presumptive 2° VPCs (Yoo et al., 2004), but further mechanistic support is lacking.

In contrast, the "sequential induction model" proposes that EGF induces only the most proximal VPC, which becomes 1°. Subsequently this presumptive 1° cell expresses DSL ligands that, via the LIN-12/Notch receptor, laterally induce neighboring VPCs to assume 2° fate (Chen and Greenwald, 2004). Accordingly, the LET-23/EGF receptor (EGFR) is necessary for 1° but not 2° fate induction (Koga and Ohshima, 1995; Simske and Kim, 1995), and pro-1° EGF and pro-2° Notch pathways together are necessary and sufficient to generate initial commitment to the 2°-1°-2° fate pattern (Greenwald, 2005; Sternberg, 2005). However, the "sequential

induction model" and the "morphogen gradient model" have yet to be mechanistically reconciled.

Additionally, via "quenching" mechanisms inappropriate pathway activities are reduced to minimize conflicting pro-1° and pro-2° signals in the same cell. Presumptive 1° cells enact programs that antagonize pro-2° signaling (Levitan and Greenwald, 1998; Yoo and Greenwald, 2005), and conversely presumptive 2° cells enact programs that antagonize pro-1° signaling (Berset et al., 2001; Berset et al., 2005; Yoo et al., 2004). For example, the LIP-1 ERK protein phosphatase is expressed in presumptive 2° lineages to quench ERK signaling. Thus, the developmental consequences of EGF activation of Ras-Raf signaling in 2° lineages are probably minimal, and pro-2° EGF activity is likely mediated through a distinct effector pathway.

The most plausible composite model for robust vulval patterning would be one that reconciles these three evidence-based models: graded morphogen signaling, sequential induction, and pathway quenching. Yet nothing is known about the mechanism of the putative pro-2° EGF signal. Despite decades of research into how a single morphogen gradient can induce multiple cell fates, there are few instances in which the mechanism of such differential inductions is understood (Piddini and Vincent, 2009).

In this study, we identify a mechanism for EGF pro-2° signaling and thus reconcile the three features of vulval patterning into a unified model. We show that during vulval patterning Ras through Raf transduces a pro-1° signal, then through the RalGEF-Ral pathway transduces a pro-2° signal. Ral signaling antagonizes Raf

and regulates the balance of 1° and 2° fates. Ral is necessary and sufficient to drive maximal Notch pro-2° activity, and the RalGEF-Ral pathway is quenched in presumptive 1° cells by restricted Ral expression. In summary, our study establishes that Ras effector utilization is controlled to signal for distinct cellular outcomes. Analogous mechanisms may therefore contribute to the distinct patterns of effector utilization that occur in different settings of mutant Ras-driven human cancers.

III. Materials and Methods

Strains

Nomenclature and methods for the culturing and handling of *C. elegans* strains are as described (Brenner, 1974; Horvitz et al., 1979). Animals were cultured at 23°C unless otherwise stated. All strains were derived from the N2 Bristol wild type.

The alleles used were LGI: *smg-1(r861)*, *hT2[qIs48](I;III)*; LGII: *lin-31(n301*lf*)*, *let-23(sa62*gf*)*, *let-23(sy1*rf*)*, *unc-4(e120)*; LGIII: *daf-2(e1370)*, *ral-1(tm2760)*, *unc-93(e1500*sd*)*, *dpy-17(e164)*, *unc-32(e189)*, *lin-12(n302*d*)*, *lin-12(n379*d*)*, *lin-12(n676*d*)*, *lin-12(n950*d*)*, *lin-12(n952*d*)*, *lin-12(n137n460*ts*)*, *glp-1(q35*sd*)*, *unc-119(ed3)*, *hT2[qIs48](I;III)*; LGIV: *eri-1(mg366)*, *him-8(e1489)*, *lin-3(n378*rf*)*, *let-60(n1046*gf*), dpy-20(e1282*ts*)*; LGX: *lin-15(n765*ts*)*. The integrated transgenes used were LGX: *syIs1* [*lin-3(*xs*)*]*;* Unmapped: *arIs92* [$P_{egl\text{-}17}$*::cfp,* $P_{ttx\text{-}3}$*::gfp,*], kuIs57 [$P_{col\text{-}10}$*::lin-45(AA)*] (Yoder et al., 2004). Extrachromosomal arrays [experimental

construct, cotransformation marker] used were: *reEx83* [P_{lin-31}*::ral-1(+),* P_{myo-2}*::gfp*], *reEx84* [P_{lin-31}*::ral-1(+),* P_{myo-2}*::gfp*], *reEx85* [P_{lin-31}*::ral-1(+),* P_{myo-2}*::gfp*], *reEx49* [P_{lin-31}*::ral-1(S31N),* P_{myo-2}*::gfp*], *reEx50* [P_{lin-31}*::ral-1(S31N),* P_{myo-2}*::gfp*], *reEx51* [P_{lin-31}*::ral-1(S31N),* P_{myo-2}*::gfp*], *reEx24* [P_{lin-31}*::ral-1(Q75L),* P_{myo-2}*::gfp*], *reEx32* [P_{ral-1}*::gfp, unc-119(+)*], *reEx33* [P_{ral-1}*::gfp, unc-119(+)*], *reEx17* [P_{lin-31}*::let-60(+),* P_{myo-2}*::gfp*], *reEx12* [P_{lin-31}*::let-60(12V), dpy-20(+)*], *reEx15* [P_{lin-31}*::let-60(12V35S), dpy-20(+)*], *reEx14* [P_{lin-31}*::let-60(12V37G),* P_{myo-2}*::gfp*].

In our hands, when cultured for an extended period of time *let-60(n1046*gf*)* was prone to acquiring genetic modifiers that altered the intensity of the hyper-induced phenotype. Therefore, we followed rigorous protocols to avoid genetic drift. All acquired or generated strains were promptly frozen. Fresh strains were parafilmed for storage up to three months. Active cultures were frequently renewed from parafilmed plates, and parafilmed plates were periodically renewed from frozen reserves. Thus, experiments throughout were performed using fresh strains.

Plasmid Construction and Transgenic Lines

ral-1(+), *ral-1(S31N)*, *ral-1(Q75L)*, *let-60(+)*, *let-60(G12V)*, *let-60(G12V,A35S)*, and *let-60(G12V,E37G)* cDNAs were generated by site-directed mutagenesis (Stratagene QuikChange) of *ral-1* cDNA yk1538b07 or *let-60* cDNA yk1438c09, respectively. Each was amplified with cDNA-specific primers containing a 5' BglII and 3' NotI site. Resulting products were digested with BglII and NotI and cloned in frame into pB255 after the *lin-31* promoter and before the *unc-54* 3'UTR (Myers and Greenwald, 2005; Tan et al., 1998). All inserts and joins were

sequenced and found to be error-free. Transgenic lines were generated by microinjecting *ral-1*- or *let-60*-containing clones (50 ng/μl) and pPD118.33 P_{myo-2}*::gfp* (20 ng/μl) or pMH86 *dpy-20 (+)* (20 ng/μl) into the hermaphrodite germline of *let-60(n1046*gf*)* or *dpy-20(e1282*ts*)* as described (Mello et al., 1991). Transgenic lines were established by selecting animals expressing GFP or displaying rescued Dpy phenotype for two generations.

A 3.5-kb fragment from the 5'- region of *ral-1* was PCR amplified from wild-type genomic DNA with the primers DJR432 (CCCAAACAAGATCGACCAGT) and DJR435 (TTCCGCTTGCTTTTTTCGATGC), digested with PstI and XbaI, and cloned in frame into pPD95.67. Transgenic lines were generated by microinjection of P_{ral-1}*::gfp* (20 ng/μl) into *unc-119(ed3)* hermaphrodites with the co-transformation marker pAZ132 *unc-119(+)* (10 ng/μl). Transgenic lines were established by selecting animals displaying rescued mobility for two generations.

ral-1(tm2760); *let-60(n1046*gf*)* Double Mutant Construction

The *ral-1(tm2760)* deletion, kindly provided by Shohei Mitani, removes nucleotides 418 -996 (numbered from position +1 of the *ral-1* initiating ATG codon), deleting part of the splice donor site. Single worm PCR of *ral-1(tm2760)* was performed as described (Williams et al., 1992). In the initial strain isolate, heterozygous *tm2760* co-segregated with a sterile mutation. Outcrossing *tm2760* and recombination in the *daf-2-dpy-17* interval failed to separate the sterile mutation from *tm2760*. *ral-1(RNAi)* did not impact fertility, even when performed in the *eri-1(mg366)* RNAi hyper-sensitive background (Kennedy et al., 2004). Consequently,

we favor the model that sterility is conferred by a mutation closely linked to *ral-1(tm2760)*. Sterile animals formed a functional vulva, so in the *let-60(n1046*gf*)* background we balanced *ral-1(tm2760)* with *unc-93(e1500*sd*) dpy-17(e164)*. We used the semi-dominant *unc-93(e1550*sd*)* Unc phenotype to distinguish tm2760/tm2760 homozygotes from *tm2760*/*unc-93 dpy-17* heterozygotes, and scored vulvas of non-Unc animals. Single worm PCR (T_m=53°, cycles=35) with the primers TZ23 (CAACAAGTCGTCCATAAAGTG), TZ24 (GGCGAAAAACGAGAAAAGAAC), and TZ25 (GAATTTTTCAGGCTTTCTGACG) confirmed the *tm2760*/*tm2760* genotype of each scored animal.

Bacterially Mediated RNAi

Bacterially mediated RNAi was performed mostly as described (Fire et al., 1998; Kamath et al., 2001; Timmons et al., 2001). Each fRNAi clone was sequenced to confirm identity. Eighty μl of bacteria was seeded on NGM agar plates containing 1 mM IPTG and 50 μg/ml carbenicillin. L4 larvae were added to the plates the following day. After 24 h, animals were transferred to new plates, and parents were removed after an additional 24 h. We consistently obtained stronger fRNAi phenotypes at 23°C, and thus all fRNAi experiments were performed at 23°C. *gfp(RNAi)* or *daf-3(RNAi)* was used as a control. *pop-1(RNAi)* was included in all experiments as a positive control for RNAi efficacy. Phenotypes were only scored if we observed 100% lethality on the *pop-1(RNAi)* plates.

The fRNAi clones used were (Kamath et al., 2003): I-3F20 (*rlbp-1*), I-7C06 (*exoc-8*), II-7J15 (*cey-1*), III-7M13 (*ral-1*), III-2I01 (*mpk-1*), III-4J14 (*lin-12*), IV-5E18

(*pld-1*), IV-3E12 (*lin-45*), IV-5H24 (*lin-3*), IV-6A16 (*let-60*), V-1I09 (*Y66H1B.3*, non-muscle filamin), X-1M03 (*daf-3*), X-1D10 (*gap-1*), X-2K11 (*rgl-1*), *gfp* (GFP subcloned into L4440/pPD129.36; kindly provided by N. Dudley.)

Vulval Induction Assay

L4 hermaphrodites were mounted as described previously in 5 mM sodium azide/M9 buffer on slides with agar pads, and visualized under DIC Nomarski optics (Sternberg and Horvitz, 1986; Sulston and Horvitz, 1977) using a Nikon Eclipse E800 microscope with a Hamamatsu C2400-07 Newvicon camera controlled by Metamorph acquisition software (Molecular Devices). WT animals scored 3.0 (3 induced VPCs). Values greater than 3.0 indicated hyper-induction, less than 3.0 under-induction.

Fluorescence Microscopy

Live animals were mounted in 2 mg/ml tetramisole/M9 buffer on slides with agar pads and visualized using a Nikon Eclipse TE2000U microscope equipped with a DVC-1412 CCD camera (Digital Video Camera Company) controlled by the Hamamatsu SimplePCI acquisition software.

IV. Results

A. *C. elegans* Contains Single RalGEF and Ral Orthologs

The *C. elegans* genome contains single RalGEF (*rgl-1*; F28B4.2) and Ral (*ral-1*; Y53G8AR.3) orthologs. *rgl-1* encodes predicted splice variants producing proteins of 860 and 880 residues that share the identical domain architecture with human Ras-GTP Association (RA) domain-containing RalGEFs: an N-terminal Ras Exchange Motif (REM), a central CDC25 homology (RasGEF) catalytic domain, and a C-terminal RA domain (Figure 2-2A). *ral-1* encodes a protein of 213 residues consisting of a GTPase domain and C-terminal membrane-targeting sequence sharing strong sequence identity (61-65%) with human RalA and RalB (Figure 2-2B). The effector binding regions of human and *C. elegans* Ral GTPases share high identity, suggesting common effector utilization (Figure 2-2C); Ral effector orthologs are also conserved in *C. elegans*. The strong conservation of the RalGEF effector pathway components suggests an important role in *C. elegans* LET-60/Ras function.

B. RGL-1-RAL-1 Antagonizes Raf in Ras-mediated Vulval Development

Genetic dissection of LET-60/Ras signaling in vulval development was instrumental in delineating the Raf-MEK-ERK pathway in mammalian cells. Consequently, we used multiple genetic approaches to dissect the role of RGL-1 and RAL-1 in LET-60-directed vulval development. In a moderately activating gain-of-function (gf) LET-60 background (*let-60* allele *n1046*; G13E mutation), we introduced *rgl-1(RNAi)* or *ral-1(RNAi)*. Unexpectedly, we found increased, rather than decreased, vulval hyper-induction (Figure 2-3A). Negative control RNAi targeting *gfp* (green fluorescent protein) and *lin-3* (encoding EGF, acting upstream) had no effect, while positive control RNAi targeting *gap-1* (encoding RasGAP, a

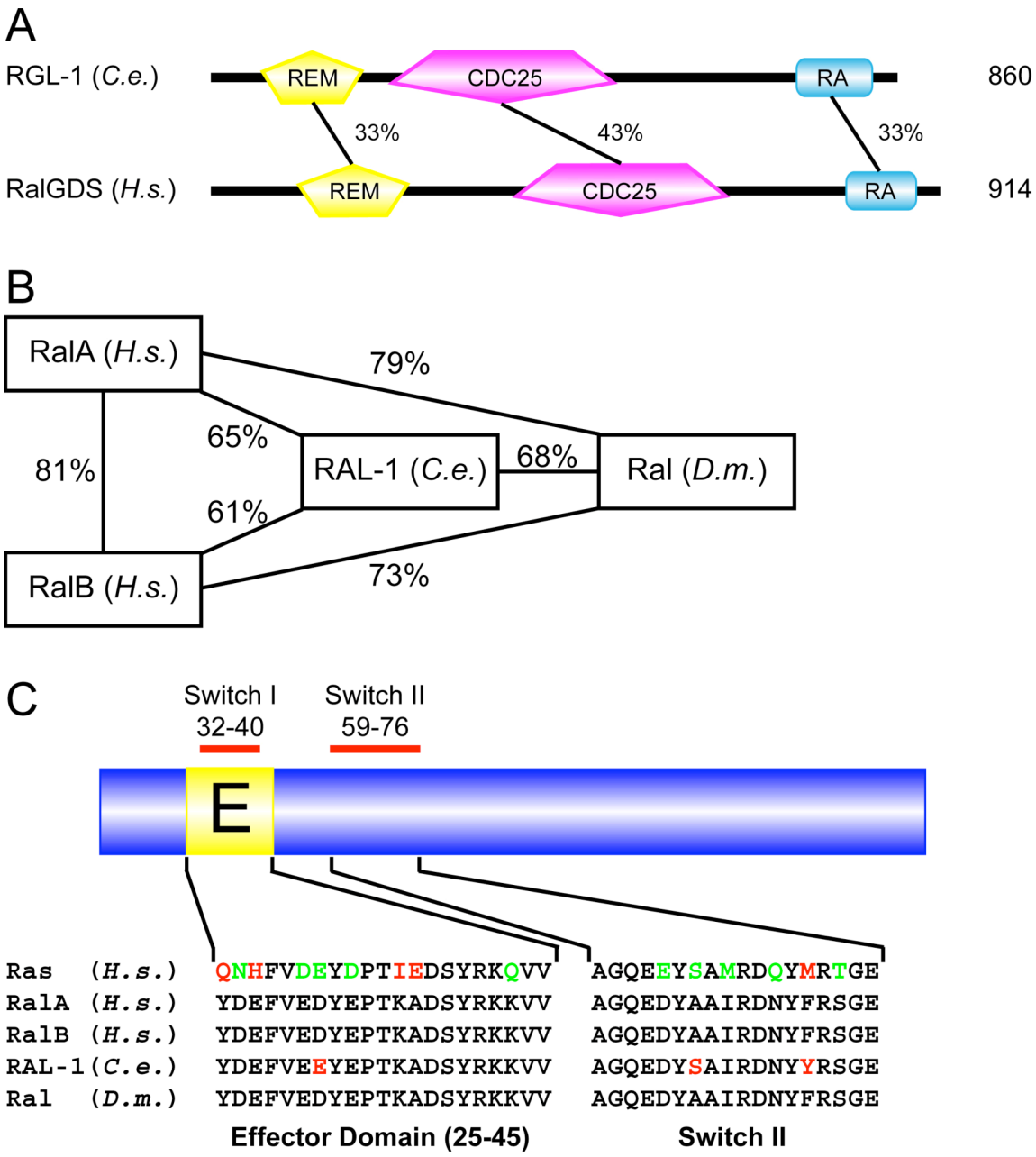


Figure 2-2. RGL-1 and RAL-1 Are Highly Conserved across Species
(A) Domain comparison of *Caenorhabditis elegans* (*C.e.*) RGL-1 with *Homo sapiens* (*H.s.*) RalGDS. Percent amino acid identity of the conserved domains is shown. Of the four human RalGEF orthologs, RalGDS exhibits the greatest sequence identity with RGL-1. **(B)** *H.s.* RalA and RalB proteins are conserved with *C.e.* RAL-1 and *Drosophila melanogastor* (*D.m.*) Ral. Percent identities are shown. **(C)** The effector interaction domains of Ral GTPases, which are contained within the switch I and switch II regions, are 95% conserved across species. The corresponding regions of human Ras (100% identical among H-, N- and K-Ras) are shown for comparison. Residue numbering is based on Ras proteins. Residues identical in all proteins are black, conservative changes are green, and non-conservative changes are red.

negative regulator of LET-60 activity) enhanced the *let-60(*gf*)* phenotype. *rgl-1* or *ral-1* knockdown in a wild-type (WT) background caused no defect (data not shown), suggesting a modulatory role for RGL-1-RAL-1 signaling.

A caveat is that the RGL-1/RAL-1-dependent phenotypes could be specific to *let-60(n1046*gf*)*, for example if the *in situ* activating mutation conferred inappropriate LET-60/Ras utilization of RGL-1. We ruled out this concern by showing that *rgl-1(RNAi)* and *ral-1(RNAi)* enhanced vulval hyper-induction due to LIN-3/EGF over-expression, an activating LET-23/EGFR mutation, and a transgene expressing activated LIN-45/Raf (Figure 2-4A-C).

To corroborate these RNAi results with an independent methodology, we also analyzed a *ral-1* deletion (Δ), *tm2760*, which removes a portion of intron 3 including the splice donor site. Presumably intron 3 splicing is blocked resulting in a null allele. *ral-1(*Δ*)* significantly enhanced the *let-60(*gf*)* hyper-induced phenotype (Figure 2-3B).

Dominant negative (dn) RAL-1(S31N), predicted to sequester and inactivate its GEF (Urano et al., 1996), should also enhance activated LET-60-driven pro-1° activity. We generated *let-60(*gf*)* animals harboring *ral-1(*dn*)* driven by the VPC-specific *lin-31* promoter (P_{lin-31}) (Tan et al., 1998). The hyper-induced vulval phenotype of *let-60(*gf*)* animals expressing RAL-1(dn) was significantly enhanced compared to non-transgenic siblings (Figure 2-3C). In contrast, VPC-specific expression of RAL-1(gf) (gain-of-function, Q75L) significantly suppressed the hyper-induced vulval phenotype of *let-60(*gf*)* animals relative to their non-transgenic siblings (Figure 2-3D), while RAL-1(gf) caused no defect in a WT background (data

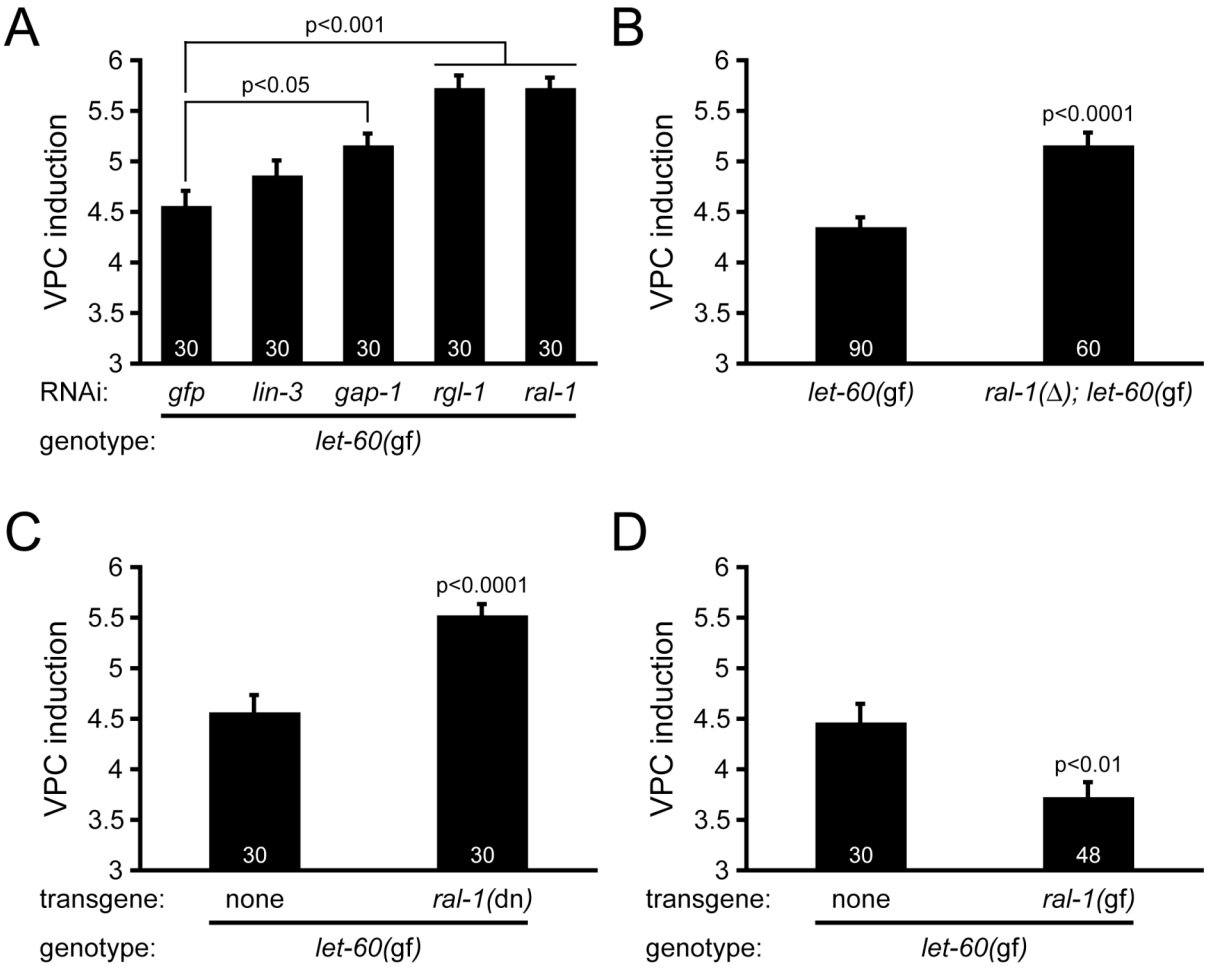


Figure 2-3. RAL-1 Antagonizes LET-60-dependent Vulval Induction
(A) *rgl-1(RNAi)* or *ral-1(RNAi)* enhanced *let-60(n1046*gf*)* hyper-induction. Controls were *gfp(RNAi), lin-3*/EGF*(RNAi)*, and *gap-1*/RasGAP*(RNAi)*. Data shown are representative of six independent assays. **(B)** *ral-1(tm2760)* enhances *let-60(n1046*gf*)*. The *n1046* single mutant was counted in nine assays, the double mutant in four. **(C)** Transgenic dominant-negative RAL-1(S31N) enhanced *let-60(*gf*)*. Two transgenes were analyzed; that shown was assayed three times, another four. **(D)** Transgenic activated RAL-1(Q75L) suppressed *let-60(*gf*)*. One transgene was assayed seven times. Y-axis is the number of VPCs induced to vulval (1° and 2°) fates. Data are the mean ± standard error of the mean (SEM). For statistical reasons single, non-pooled assays are shown, and white numbers represent animals scored therein. Statistics were calculated by Kruskal-Wallis, Dunn test (A) or Mann-Whitney test (B-D).

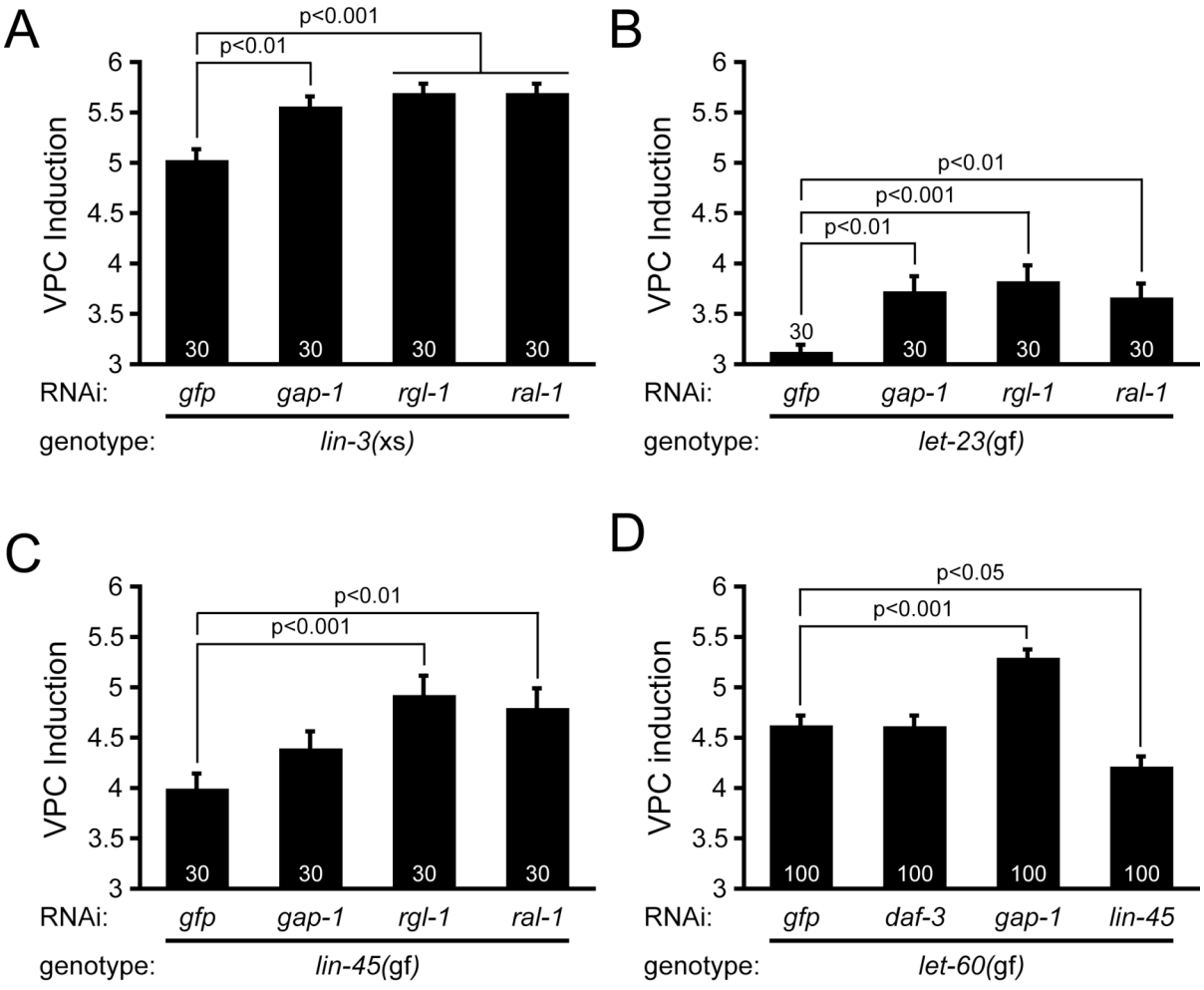


Figure 2-4. LET-60/Ras-RGL-1-RAL-1 Antagonizes LET-60/Ras-LIN-45/Raf
(A-C) *rgl-1(RNAi)* or *ral-1(RNAi)* enhances vulval hyper-induction conferred by *syIs1* (*lin-3(*xs*)*), *let-23(sa62*gf*)*, and *kuIs57* (*lin-45(*gf*)*). The negative control is *gfp(RNAi).* As expected, the positive control *gap-1(RNAi)* enhances at or upstream of *let-60,* but not downstream. Data shown are representative of five independent assays. **(D)** *daf-3(RNAi)*, a negative control, is equivalent to *gfp(RNAi)* in our assay. *gap-1*/RasGAP*(RNAi)* enhanced while *lin-45*/Raf*(RNAi)* suppressed the *let-60(n1046*gf*)* hyper-induced vulval phenotype. The *lin-45* containing feeding RNAi clone (Kamath et al., 2003) targets both the *lin-45* 3' UTR, and *smg-7* coding sequences. Data shown are representative of three independent assays. Y-axis is the number of VPCs induced to vulval (1° and 2°) fates. Data are the mean ± standard error of the mean (SEM). For statistical reasons single, non-pooled assays are shown, and white numbers represent animals scored therein. Statistics were calculated by Kruskal-Wallis, Dunn test.

not shown). Control VPC-specific expression of WT RAL-1 did not alter the *let-60(*gf*)* phenotype, indicating that our VPC expression system is phenotypically neutral (data not shown).

Taken together, these data support four conclusions. First, RGL-1 and RAL-1 antagonize the canonical Raf-MEK-ERK pro-1° signal. Second, RGL-1 and RAL-1 function comparably in vulval patterning and likely comprise a RGL-1-RAL-1 signaling module. Third, RGL-1 and RAL-1 function cell autonomously in VPCs. Fourth, neither loss nor gain of RGL-1 or RAL-1 function in a WT background perturbed vulval patterning, suggesting that RGL-1 and RAL-1 are not part of core pro-1° or pro-2° induction pathways, but rather are modifiers of LET-60/Ras stimulated vulval patterning signals. Other comparable pathway modifiers, both positive and negative, have been identified in sensitized genetic screens. Perturbation of these genes caused no phenotype alone, but collectively they exert a profound influence on vulval patterning (Berset et al., 2001; Berset et al., 2005; Sundaram, 2006; Yoo et al., 2004; Yoo and Greenwald, 2005).

C. RAL-1 Signals through Multiple Effectors to Antagonize Ras-Raf Signaling

Previously RAL-1 was shown to function redundantly with the Rap ortholog RAP-1 in epithelial morphogenesis, though neither Ras nor Raf was characterized in this process. RAP-1 and RAL-1 cooperate to control proper junctional localization of α-catenin. In this capacity RAL-1 uses as effectors the Sec5 and Exo84

components of the exocyst complex (Frische et al., 2007). We therefore determined which effector(s) mediate RAL-1 antagonism of Raf signaling.

Ral GTPases signal through functionally diverse effectors (Bodemann and White, 2008). We evaluated available putative *C. elegans* orthologs of Ral effectors EXOC-84 (Exo84), RLBP-1 (RalBP1), Y66H1B.3 (non-muscle filamin), PLD-1 (Phospholipase D), and CEY-1 (ZONAB). RNAi of three different putative Ral effectors, RLBP-1, Y66H1B.3 (non-muscle filamin), and PLD-1, enhanced the *let-60(*gf*)* hyper-induced phenotype (Figure 2-5). However, in contrast to the redundant RAL-1/RAP-1 morphogenetic activity, loss of an exocyst complex subunit, EXOC-84, had no effect in vulval patterning. Suppression of no single effector was quantitatively equivalent to suppression of RGL-1 or RAL-1 activity, suggesting that multiple effectors function cooperatively downstream of RAL-1 in vulval patterning.

D. LET-60/Ras Mediates Genetically Separable Pro-1° and Antagonistic Signals

In *Drosophila* the Rap family of Ras-related small GTPases has been implicated in RalGEF activation (Mirey et al., 2003; Rodriguez-Viciana et al., 2004). However, *rap-1(RNAi)* or *rap-2(RNAi)* did not alter the *let-60(*gf*)* phenotype (not shown), suggesting that Raps are not required for the RGL-1-RAL-1 signal. RGL-1 was identified previously in a yeast two-hybrid screen with activated LET-60 bait, but further characterization was not pursued (Shibatohge et al., 1998). We hypothesize that LET-60/Ras binds and activates RGL-1 to antagonize the Ras-Raf pro-1° signal.

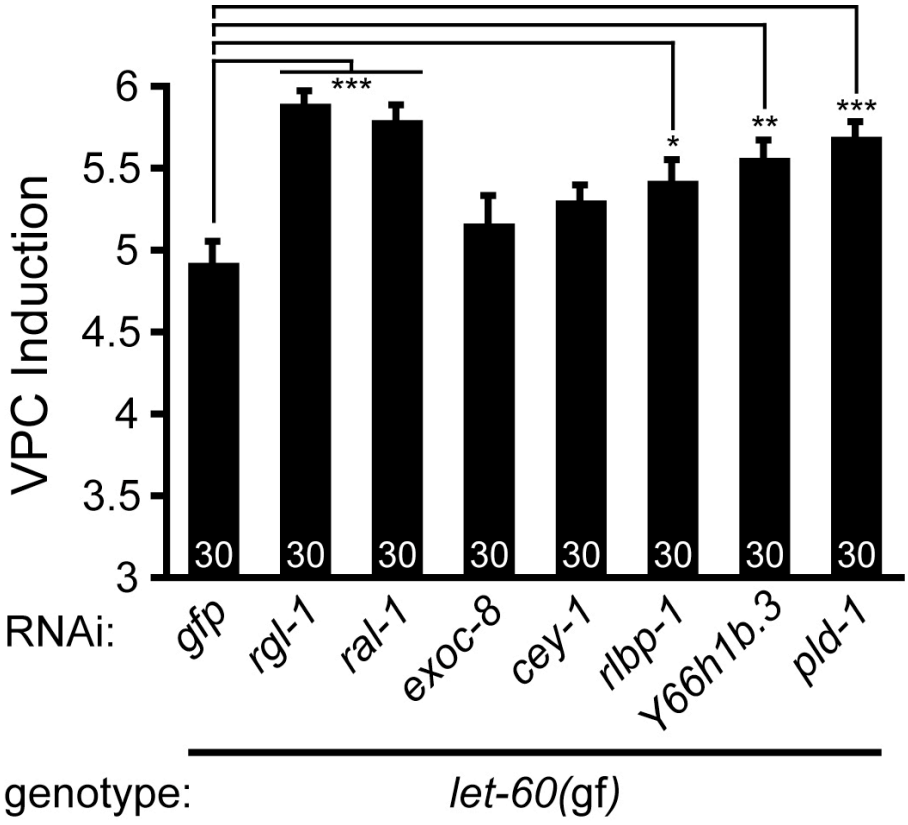


Figure 2-5. LET-60/Ras-RGL-1-RAL-1 Signals through Multiple Effectors to Antagonize LET-60/Ras-LIN-45/Raf Signaling

RNAi targeting several potential Ral effectors (*rlbp-1*/RalBP1, *Y66h1b.3*/non-muscle filamin, and *pld-1*/PLD-1) enhances the *let-60(n1046*gf*)* hyper-induced vulval phenotype. RNAi targeting the following tested negative in this assay: *exoc-8*/Exo-84, *cey-1*/Zonab, *pdk-1*/PDK-1. Data shown are representative of three independent assays. Y-axis is the number of VPCs induced to vulval (1° and 2°) fates. Data are the mean ± standard error of the mean (SEM). For statistical reasons single, non-pooled assays are shown, and white numbers represent animals scored therein. Statistics were calculated by Kruskal-Wallis, Dunn test.

LET-60/Ras activation of LIN-45/Raf is an essential event for vulval induction, so we could not directly assess the necessity of LET-60 for RGL-1 activation. To bypass this complication we used animals harboring the *lin-31(n301)* null mutation ("0"), which causes moderate hyper-induction (Miller et al., 1993). RNAi of *let-60*, *rgl-1* or *ral-1* enhanced the *lin-31(*0*)* hyper-induced phenotype (Figure 2-6A), suggesting that LET-60-RGL-1-RAL-1 functions parallel to or downstream of LIN-31. We subjected *lin-31(*0*)* animals to RNAi targeting *lin-45*/Raf and *mpk-1*/ERK and found no effect on hyper-induction, suggesting that *lin-31(*0*)* is independent of upstream pro-1° signaling activity. *lin-45* and *mpk-1* RNAi were validated separately for activity (Figure 2-4D and not shown). Our results suggest that LET-60 has dual functions, first, to transduce the canonical Ras-Raf pro-1° signal, and second, to bypass Ras-Raf pro-1° activity with an antagonistic Ras-RalGEF-Ral signal.

E. Ectopic LET-60/Ras Is Sufficient to Induce RGL-1-RAL-1 Activity

LET-60 shares 73-77% identity with human Ras proteins, with 100% identity in the core effector-binding domain (32-40). To assess whether Ras is sufficient to activate RGL-1, we used mutationally activated LET-60 (*let-60(12V)*) with missense mutations that result in differentially impaired effector binding. The E37G mutation retains effective interaction with RalGEF but not Raf or PI3K, whereas the T35S mutation retains Raf but not PI3K or RalGEF binding (White et al., 1995). We generated otherwise WT animals harboring transgenes driving VPC-specific expression of *let-60(12V)* (general gf) or *let-60(12V,35S)* (Raf gf). As expected by their ability to bind Raf, both transgenes caused a hyper-induced phenotype (Figures

2-6C and 2-6D). In contrast, transgenic VPC-expressed *let-60(12V,37G)* (RalGEF gf) significantly suppressed the hyper-induced phenotype of *let-60(*gf*)* animals compared to non-transgenic siblings (Figure 2-6B), equivalent to the effect of *ral-1(*gf*)* (above) and consistent with RalGEF-selective activity. Control VPC-specific expression of *let-60(+)* in the *let-60(*gf*)* background caused no phenotype (data not shown).

While the H-Ras(12V,37G) effector-binding mutant is impaired in its ability to activate PI3K and Raf, the mutant protein still retains the ability to bind other Ras-binding proteins in addition to RalGEF (Kelley et al., 2001). Therefore, we showed that the LET-60(12V,37G) phenotype is RGL-1- and RAL-1-dependent (Figure 2-6B), demonstrating that the LET-60(12V,37G) phenotype is not due to signaling through other effectors. Thus, we conclude that the LET-60-RGL-1-RAL-1 pathway is a bona fide vulval signaling module.

F. RAL-1 Contributes to the 1°/2° Fate Decision

To evaluate directly whether disruption of *ral-1* perturbs cell fate specification, we used a P_{egl-17}*::cfp-LacZ* transgene as a reporter of 1° cell fate (Yoo et al., 2004). Notch-dependent lateral signal normally prevents formation of neighboring 1° lineages. However, when the 1°:2° signaling balance is genetically disrupted to favor 1° fate, the frequency of neighboring 1° lineages increases (Berset et al., 2001; Berset et al., 2005; Yoo et al., 2004). In a *let-60(*gf*)* background, *ral-1(RNAi)* increased significantly the number of adjacent CFP-positive lineages (Figures 2-7A-F). *daf-3(RNAi)* rather than *gfp(RNAi)* was used as a negative control (Figure 2-4D).

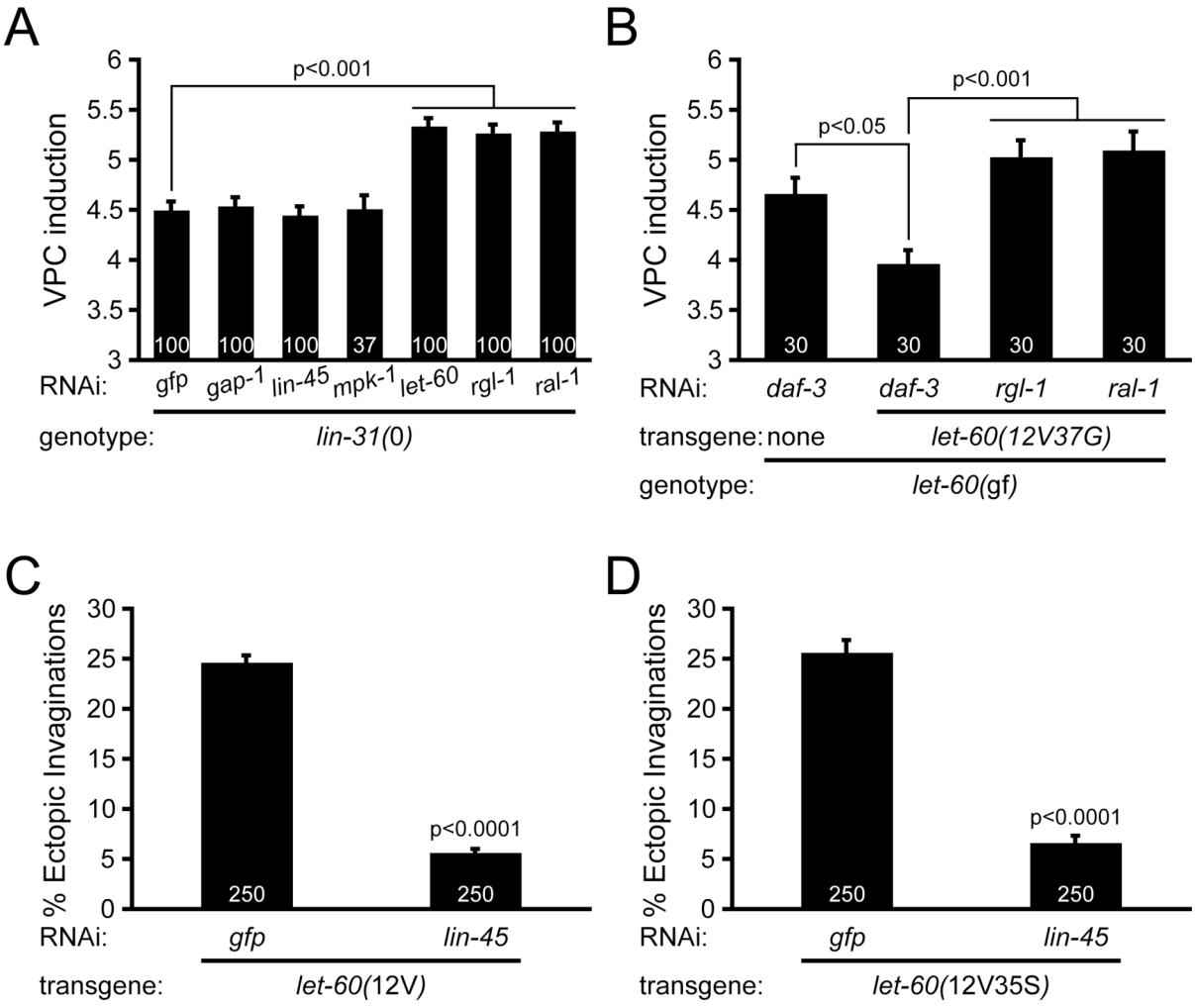


Figure 2-6. Ras-RGL-1-RAL-1 Bypasses Ras-Raf
(A) *let-60-, rgl-1-,* and *ral-1*-directed RNAi enhanced the hyper-inducing *lin-31(n301)*. *gfp(RNAi), gap-1(RNAi), lin-45(RNAi),* and *mpk-1(RNAi)* controls were negative. Data shown are representative of three independent assays. **(B)** Transgenic activated LET-60(12V,37G) (RalGEF selective) suppressed *let-60(n1046*gf*)* compared to non-transgene bearing siblings, and was RGL-1 and RAL-1 dependent. Two transgenes were analyzed three times each. Y-axis is the number of VPCs induced to 1° and 2° cell fates. Data are the mean ± SEM. For statistical reasons single, non-pooled assays are shown, and white numbers represent animals scored therein. **(C)** Transgenic activated LET-60(12V) (general gf) or **(D)** activated LET-60(12V35S) (Raf-selective) induced ectopic, LIN-45/Raf-dependent pseudovulvae. Average percent animals with ectopic pseudovulvae in three independent assays are shown ± SEM. In *let-60(12V)* the number of ectopic invaginations out of the total per assay was, for gfp*(RNAi)* 13/50, 24/100, and 24/100 and for *lin-45(RNAi)* 3/50, 6/100, and 5/100. In *let-60(12V35S)*, for *gfp(RNAi)* 12/50, 25/100, and 28/100 and for *lin-45(RNAi)* 3/50, 8/100, and 6/100. White numbers represent pooled total animals scored. Statistics were calculated by Kruskal-Wallis, Dunn test (A and B) or Fisher's Exact test (C and D).

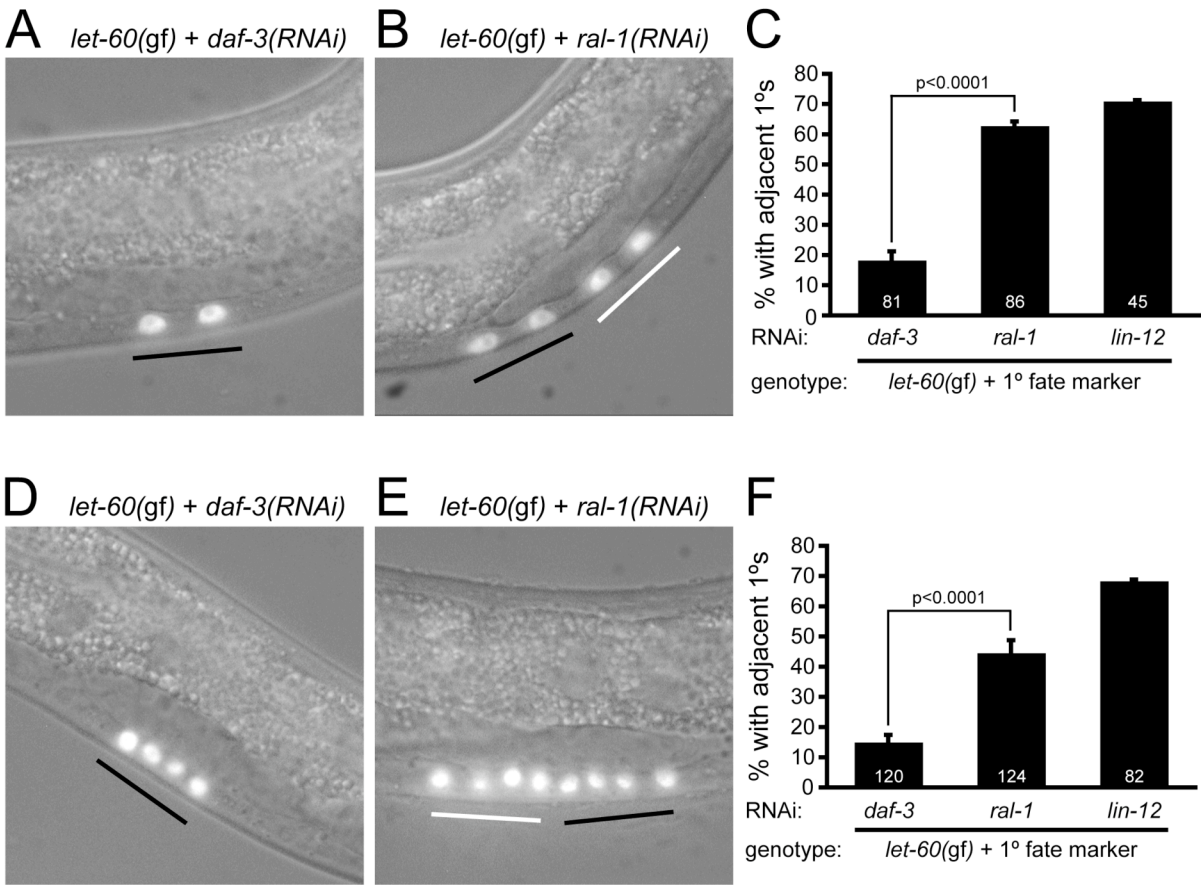


Figure 2-7. Loss of RAL-1 Permits Adjacent 1° Cells

(A, B, D and E) Expression of P_{egl-17}*::cfp-lacZ* in VPC daughters. Overlaid DIC and CFP fluorescence images of *let-60(n1046*gf*); daf-3(RNAi)* **(A and D)** and *let-60(*gf*); ral-1(RNAi)* **(B and E)** at the Pn.px stage **(A and B)** and Pn.pxx stage **(C and D)**. The black bar indicates P6.p descendents and the white bar indicates P5.p or P7.p descendents. **(C and F)** Percent L3 larvae with CFP–positive lineages neighboring the P6.p lineage (P5.p or P7.p derived) at the Pn.px stage **(C)** and Pn.pxx stage **(F)** in the *let-60(*gf*); arIs92* (P_{egl-17}*::cfp-lacZ)* background. Shown are average percentages of animals with adjacent 1° cell fate from three independent assays ± SEM. In the Pn.px stage **(C)**, the numbers of adjacent 1° cells out of the total per assay were, for *daf-3(RNAi)* 3/25, 6/26, and 6/30, for *ral-1(RNAi)* 7/27, 19/29, and 18/30, and for *lin-12(RNAi)* 12/17 and 20/28. In the Pn.pxx stage **(F)**, the numbers of adjacent 1° cells out of the total per assay were, for *daf-3(RNAi)* 5/40, 8/39, and 5/41, for *ral-1(RNAi)* 15/40, 15/28, and 24/56, and for *lin-12(RNAi)* 20/29 and 36/53. White numbers represent pooled total animals scored. Statistics were calculated by Fisher's Exact test.

We conclude that RAL-1 activity promotes 2° fate at the expense of 1° fate.

G. RAL-1 Cooperates with Notch to Specify 2° Vulval Fate

1° and 2° fates are mutually antagonistic, so a putative RGL-1-RAL-1 pro-2° signaling pathway that cooperates with Notch pro-2° signaling is consistent with the observed Ras-RGL-1-RAL-1 antagonism of the Ras-Raf pro-1° signal. We evaluated this model using sensitized dominant activated (d) LIN-12/Notch backgrounds. *lin-12(n302*d*)* and *lin-12(n379*d*)* mutant animals have two features critical for our study. First, they lack a functional AC, the source of EGF, and second their activated pro-2° signal is relatively weak, and thus potentially sensitive to further stimulation (Greenwald et al., 1983). Importantly, since there is no AC, the Notch activity assayed is likely to be LIN-3/EGF-independent. VPC-specific expression of activated *ral-1(*gf*)* significantly enhanced *lin-12(*d*)* excess 2° cell phenotypes (Figures 2-8A-D). Ectopic *ral-1(*gf*)* also enhanced *glp-1(q35*d*)*/+ (Figure 2-8E); *glp-1* encodes the second *C. elegans* Notch receptor (Mango et al., 1991). RAL-1 is therefore sufficient to promote Notch pro-2° activity.

To determine whether RAL-1 is also necessary for LIN-12/Notch function, we used *ral-1(RNAi)* in weakly activated *lin-12(n302*d*)* mutant animals, as well as the moderately activating *n676*d and strongly activating *n950*d and *n952*d mutant animals. In no case did we observe *ral-1(RNAi)* suppression of the *lin-12(*d*)* excess 2° phenotype (data not shown). We hypothesize that RAL-1 is required only for the EGF pro-2° signal, and not the Notch pro-2° activity per se, and thus loss of RAL-1 has no consequences in the absence of EGF signal.

To evaluate this model we determined RAL-1 necessity for LIN-12/Notch pro-2° function under EGF-dependent conditions. A *lin-12*/Notch hypomorphic allele confers mildly compromised 2° induction, yet the AC is still present and a single vulval invagination forms (Sundaram and Greenwald, 1993). In this background, *ral-1(RNAi)* caused modest but significant losses of 2° lineages and increased morphogenetic defects (Table 2-1). We propose that *ral-1* is necessary for full LIN-12/Notch pro-2° activity, but only under EGF-dependent conditions. These observations suggest that RAL-1 contributes to an EGF signal that promotes 2° fate.

H. EGF Levels Insufficient for 1° Induction Can Induce 2° Fate in a RAL-1-dependent Manner

EGF was shown previously to be sufficient to induce 2° cells in the absence of neighboring 1° cells, arguing that there exists an EGF pro-2° signal (Katz et al., 1995; Katz et al., 1996; Sternberg and Horvitz, 1986, 1989). To examine a putative RAL-1 function in propagating the pro-2° EGF signal, we used two reagents to develop a robust EGF pro-2° signaling assay. First, *lin-12(n379*d*)*, as described above, is a weakly activating Notch mutation that abolishes AC development in ~90% of animals and weakly induces an ectopic 2° phenotype (Greenwald et al., 1983). Second, to titrate EGF levels with temperature, we added to the *lin-12(*d*)* background the temperature sensitive *lin-15(n765*ts*)* mutation, which at 15°C causes no ectopic 1° induction but at 25°C is strongly hyper-induced via ectopic EGF expression. *lin-15* encodes components of a transcriptional regulatory complex that

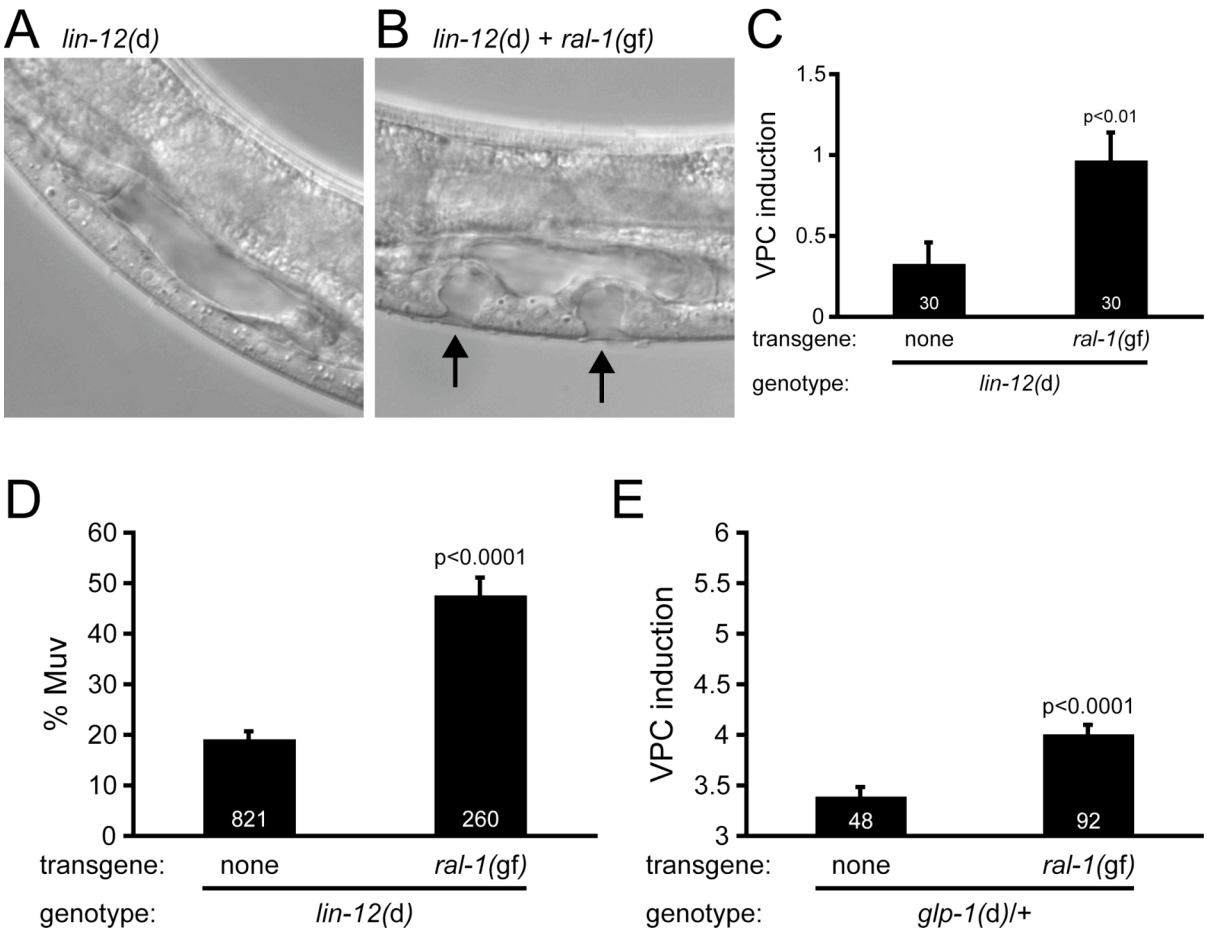


Figure 2-8. RAL-1 Is Sufficient to Promote LIN-12/Notch Pro-2° Activity
(A and B) DIC micrographs of late L4 stage **(A)** *lin-12(n302*d*)* and **(B)** *lin-12(n302*d*)* + *ral-1(Q75L)* animals. Arrows indicate ectopic 2° cells. Anterior is left and ventral is down. **(C)** Transgenic activated RAL-1(Q75L) enhanced 2° cell induction of activated *lin-12(n302*d*)*. Two transgenes were assayed four times each. Y-axis is mean number of VPCs induced to 2° cell fate ± SEM. For statistical reasons a single non-pooled assay is shown, and white numbers represent animals scored therein. **(D)** Transgenic activated RAL-1(Q75L) enhanced the Muv phenotype of activated *lin-12(n379d)*. Shown are average percentages of adult animals with a hyper-induced phenotype in four independent assays ± SEM. For *lin-12(n302*d*)* alone the numbers of hyper-induced hermaphrodites out of the total per assay were 39/169, 35/178, 38/211, and 43/263, and for *lin-12(n379*d*)* + transgenic activated RAL-1Q75L bearing siblings were 36/63, 22/52, 25/58, and 42/87. Another transgene was assayed three times. White numbers are pooled total animals scored. **(E)** Transgenic activated RAL-1(Q75L) enhanced ectopic 2° cell induction of activated *glp-1(q35*d*)/+* L4 animals. Results are from three pooled assays. Y-axis is the number of VPCs induced to vulval (1° and 2°) fates ± SEM. White numbers are animals scored in the assay shown. Statistics were calculated by Mann-Whitney test (C and E) or Fisher's Exact test (D).

Table 2-1. RAL-1 is Necessary for Full LIN-12/Notch Pro-2° Activity

Row	Genotype	% abnormal vulva	% loss of 2º fate	n
1	*lin-12(*lf, ts*)*; *gfp(RNAi)*	33.3	1.0	504
2	*lin-12(*lf, ts*)*; *ral-1(RNAi)*	46.1**	4.1*	434
3	*lin-12(*lf, ts*)*; *lin-12(RNAi)*	65.2****	14.8****	310

The *lin-12(n137n460)* temperature sensitive hypomorph is sensitive to loss of RAL-1. *ral-1(RNAi)* caused significant loss of 2° lineages and increased incidence of morphologically abnormal vulvae. Results are from three pooled assays (*p<0.05, **p<0.01, ****p<0.0001). Statistics were calculated by Fisher's Exact test.

represses LIN-3/EGF expression outside of the AC, in the epithelia surrounding the VPCs (Cui et al., 2006).

We compared the *lin-12(*d); *lin-15(*ts*)* double mutant to *lin-12(*d*)* and *lin-15(*ts*)* single mutant strains from 15° to 18°C (Figure 2-9A). *lin-12(*d*)* is not temperature sensitive; at all temperatures ~10% of animals had a normal AC/vulva and animals averaged ~0.4 ectopic invaginations that we judged to be 2° based on morphological criteria (the distal 2° lineage cells adhere to the cuticle, while the proximal cells invaginate) (Katz et al., 1995). In *lin-15(*ts*)* single mutant animals, we observed no and rare ectopic vulval induction at 15°C and 16°C respectively; however, since all animals had an AC they formed normal vulvae. But *lin-15(*ts*)* ectopic induction greatly increased at 17°C and 18°C, and these invaginations contained combined 1° and 2° lineages typical for ectopic pro-1° signaling. To better contrast the *lin-15(*ts*)* single mutant ectopic pro-1° phenotype with the double mutant *lin-12(*d*)*; *lin-15(*ts*)* pro-2° phenotype, below, we show only ectopic pseudovulvae induced in the *lin-15(ts)* single-mutant background, and excluded WT vulvae (Figure 2-9A, red).

Strikingly, in the double mutant strain we observed strong synergy at 15°C and 16°C ($P<0.00001$ for both), temperatures at which *lin-15(*ts*)* alone was not sufficient to induce ectopic 1° invaginations (Figure 2-9A). By morphology these excess invaginations were strictly 2°, indicating that sub-threshold EGF activity in a sensitized background induced large numbers of 2° cells. To verify that the observed *lin-15(*ts*)* effect was EGF-dependent, we targeted *lin-3*/EGF with RNAi and observed suppression of *lin-15(*ts*)* canonical and synergistic phenotypes (data not shown).

Thus we demonstrate that the putative EGF pro-2° signal cooperates with the Notch pro-2° signal to specify 2° fate, a property predicted to increase fidelity of vulval patterning. Furthermore, we have precisely controlled EGF input into 2° fate induction.

A parsimonious working model posits that in presumptive 2° cells, EGF-activated Ras signals preferentially through RGL-1 rather than Raf. A prediction of this model is that RAL-1 activity is necessary for full LIN-3/EGF pro-2° signaling activity. Our new system for studying this LIN-3 signaling property allowed us to analyze sufficient numbers of animals to evaluate our model. We subjected *lin-12(*d*)*; *lin-15(*ts*)* animals grown at 16°C to *gfp-*, *ral-1-*,and *lin-12*-directed RNAi (Figure 2-9B). Importantly, loss of *ral-1* significantly suppressed the level of 2° hyper-induction. Loss of *lin-12* dramatically reduced ectopic 2° induction ($P<0.001$). As an internal control for *lin-12(RNAi)* efficacy, we observed dramatic suppression of the *lin-12(*d*)* absent-AC defect (from 13.2% with *gfp*(*RNAi)* to 76.5% with *lin-12(RNAi)*). Together, our results implicate that the Ras-RalGEF-Ral pathway specifically facilitates the EGF pro-2° signal.

I. RAL-1 Expression is Consistent with Pro-2° Activity

Transgenic embryos harboring a *ral-1* promoter-driven *gfp* fusion construct showed broad GFP expression, which may reflect the endogenous RAL-1 expression pattern. Post-embryonic expression was gradually restricted to excretory canals, a small number of head and tail neurons, and vulval lineages. Vulval GFP expression was spatiotemporally dynamic. Prior to EGF induction, GFP was

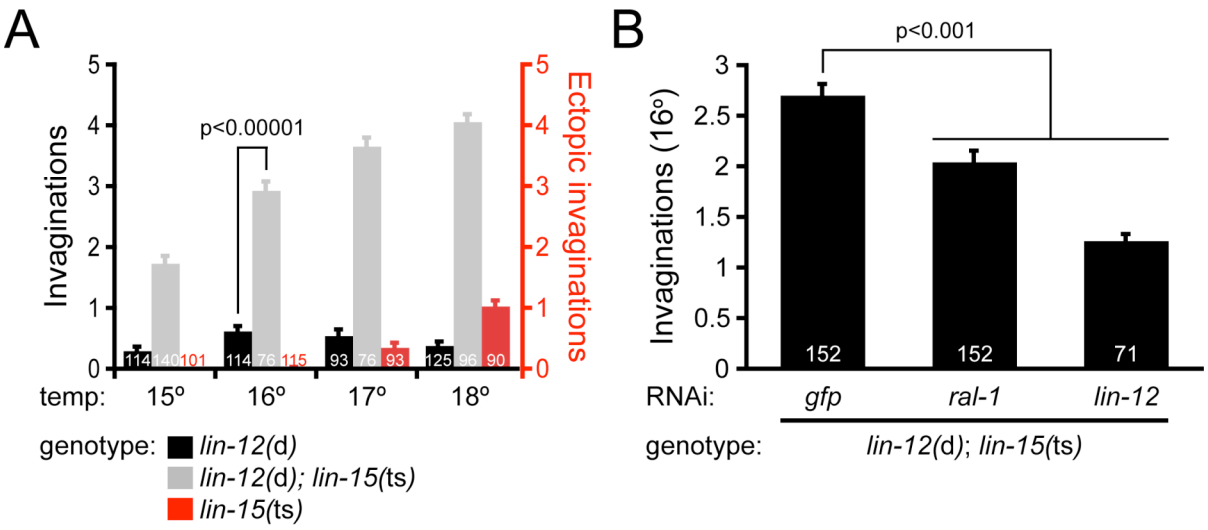


Figure 2-9. LIN-3/EGF Signals through LET-60/Ras-RGL-1-RAL-1 to Promote 2° Fate
(A) A comparison of *lin-12(n379*d*)* alone (black bars), *lin-12(n379*d); *lin-15(n765*ts*)* (gray bars) and *lin-15(n765*ts*)* alone (red bars), all grown on *gfp(RNAi)*. Animals were grown at 15˚,16˚,17˚ or 18˚C. Total vulval invaginations (left, black Y axis for black and gray columns), or ectopic pseudovulval invaginations (right, red Y axis for red columns) were scored. A single assay was performed at each temperature. **(B)** *ral-1(RNAi)* or *lin-12(RNAi)* suppressed invaginations induced by *lin-12(n379*d*); lin-15(n765*ts*)* at 16°C. Data shown are representative of three independent assays. Data are the mean ± SEM. For statistical reasons single, non-pooled assays are shown, and white numbers represent animals scored therein. Statistics were calculated by Mann-Whitney test (A) or Kruskal-Wallis, Dunn test (B)

expressed in all VPCs (Figure 2-10A), but at the time of induction, GFP was restricted to P5.p, P6.p and P7.p, cells receiving the EGF signal (Figure 2-10B). Soon thereafter expression was extinguished in the presumptive 1° cell (P6.p), persisted strongly in presumptive 2°s (P5.p and P7.p), and was faintly restored in presumptive 3°s (Figure 2-10C). Further dynamic expression changes were seen in later vulval development (Figures 2-10D-F and Table 2-2).

We propose that as an integral part of vulval patterning, LET-60/Ras switches effectors from pro-1° LIN-45/Raf output in presumptive 1° cells to pro-2° RGL-1-RAL-1 output in presumptive 2° cells. Consistent with this model is our observation that RAL-1 expression following initial induction is quickly restricted to presumptive 2°s. Since LIP-1/ERK phosphatase quenches the ERK signal in presumptive 2° cells, perhaps therein RGL-1-RAL-1 is the predominant Ras effector output.

The early vulval RAL-1 expression pattern mirrors that of LIP-1 (Berset et al., 2001), suggesting that both RAL-1 and LIP-1 are precociously present to influence interpretation of the initial EGF inductive signal. If so, the Ras-RalGEF-Ral pro-2° response to EGF is expected to conflict with the Ras-Raf pro-1° response. Likewise, the Ras-Raf pro-1° response would be blunted in presumptive 1° cells by early LIP-1/ERK phosphatase expression. Therefore, rapid exclusion of both LIP-1 and RAL-1 proteins from the presumptive 1° cell is necessary for maximal Ras-Raf pro-1° activity. Consistent with this model, *rgl-1(RNAi)* or *ral-1(RNAi)* suppressed the under-induced phenotype conferred by hypomorphic mutations in *lin-3*/EGF or *let-23*/EGFR (Figures 2-11A and 2-11B), and comparable suppression was observed with loss of LIP-1 (Berset et al., 2001). We argue that loss of either RAL-1 or LIP-1

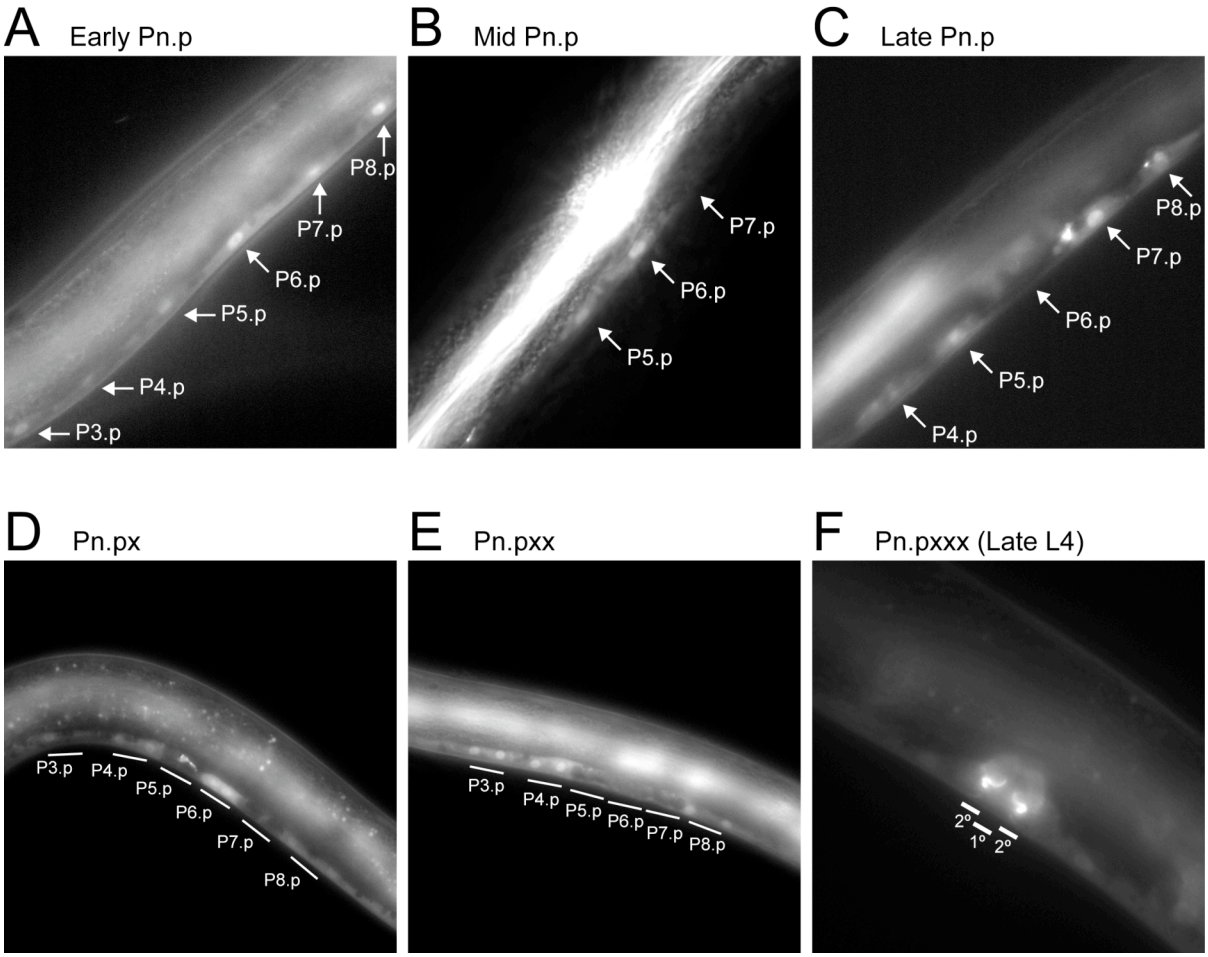
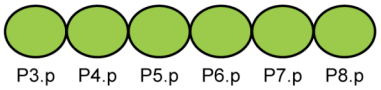
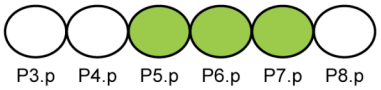
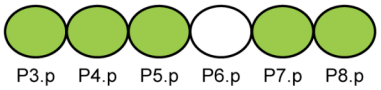
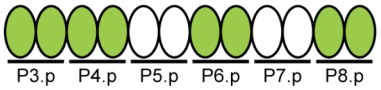
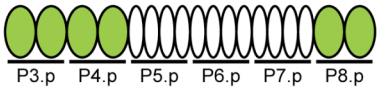
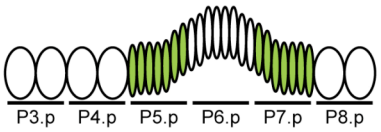


Figure 2-10. $P_{ral\text{-}1}$*::gfp* is Dynamically Expressed during Vulval Development
$P_{ral\text{-}1}$*::gfp* is expressed in all VPCs before induction, but is gradually restricted to, first, EGF-induced presumptive 1° and 2° VPCs, and then 2° cells. **(A)** Early Pn.p stage before induction. **(B)** Mid Pn.p stage. The background glow is strong excretory canal expression. The P7.p nucleus was GFP-positive, but was out of the plane of focus. **(C)** Late Pn.p stage. GFP contained a nuclear localization signal and thus localized to nuclei. **(D)** Pn.px stage. **(E)** Pn.pxx stage. As expected, we observed two rather than four nuclei in the 3° lineages, because the 3° VPC divides once and fuses with the hypodermis (3° is a non-vulval fate) (Sulston and Horvitz, 1977). By DIC four nuclei were present in each of the vulval lineages where GFP was absent (data not shown). **(F)** Late L4 (morphogenesis). Expression was clearly restricted to a sizable set of 2° lineages, but we did not determined whether expression is in all 2° cells.

Table 2-2. RAL-1 Expression Pattern Details

Time/Benchmark	GFP/DIC	Expression
Early Pn.p Before gonad extension and VPC Induction		All 6 VPCs
Mid Pn.p Gonad covers all 6 VPCs		P5, P6, P7
Late Pn.p VPCs just starting to divide		P3, P4, P5, P7, P8 (No P6!)
Pn.px		P3, P4, P6, P8 (No P5 or P7)
Pn.pxx		P3, P4, P8 (3° only)
Pn.pxxx (Late L4) Morphogenesis		Sub-2° Lineages

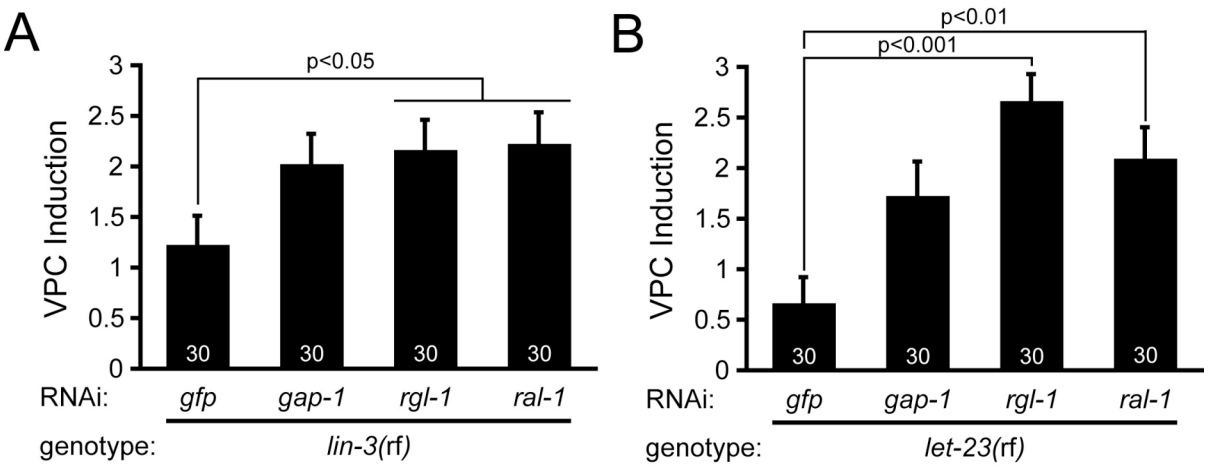


Figure 2-11. Loss of the RGL-1-RAL-1 Signal Promotes the Pro-1° LET-60/Ras-LIN-45/Raf Signal

(A and B) *rgl-1(RNAi)* or *ral-1(RNAi)* suppresses the vulval under-induction conferred by hypomorphic mutations (reduction-of-function; "rf") in *lin-3(n378*rf*)* or *let-23(sy1*rf*)*. Data shown are representative of three independent assays. Y-axis is the number of VPCs induced to vulval (1° and 2°) fates. Data are the mean ± standard error of the mean (SEM). For statistical reasons single, non-pooled assays are shown, and white numbers represent animals scored therein. Statistics were calculated by Kruskal-Wallis, Dunn test.

strengthens the initial pro-1° inductive event in presumptive 1° cells, and thus rescues compromised EGF pro-1° signaling. Hence, RAL-1 and LIP-1 cooperate as a programmed switch to toggle Ras output from Raf to RGL-1 in presumptive 2° cells.

V. Discussion

A. RGL-1-RAL-1 Provides a Mechanistic Key to Interpretation of the EGF Morphogen Gradient

The continually expanding number of functionally diverse effectors raises the issue of how Ras signaling output is controlled through dynamic spatial and temporal effector utilization to orchestrate its complex biology in normal and neoplastic cells. We describe mechanisms whereby a balance of redirected effector signal output and pathway quenching can bring two antagonistic pathways into harmony, with each faithfully promoting divergent fates in response to the same initial patterning signal. This general patterning reinforcement/fidelity mechanism may prove to be widespread in metazoan development.

The molecular mechanisms of EGF induction of 1° fate and consequent 1°-dependent Notch induction of 2° fate are well characterized. Additionally, a graded EGF receptor signal has been shown to exist, but direct EGF signaling from the AC is sufficient but not necessary for 2° fate induction (Katz et al., 1995; Katz et al., 1996; Koga and Ohshima, 1995; Simske and Kim, 1995; Sternberg and Horvitz,

1986, 1989). The mechanism by which the pro-2° EGF receptor signal is propagated was previously unknown.

We incorporate the insights from our study of Ral signaling into a new model in which we reconcile prior models of graded morphogen signaling, sequential induction, and signal quenching (Figure 2-12). We mechanistically validated the "graded morphogen model" and show that Ras effector switching is critical to the relationship between Ras and Notch. In the AC-proximal VPC (P6.p) EGF activates Ras and the ERK MAPK cascade to induce 1° fate, which by stimulating production of Notch ligands in turn induces 2° fate in neighboring VPCs. In presumptive 2° cells the Raf pro-1° signal is rapidly quenched by 2°-specific expression of LIP-1/ERK phosphatase (Berset et al., 2001) and other negative regulators (Berset et al., 2005; Yoo et al., 2004; Yoo and Greenwald, 2005). Instead, Ras signals through RGL-1 to promote 2° fate. Thus, the utilization of the RGL-1-RAL-1 signaling module is a critical feature of the differential response of cells across the EGF gradient. Such pathway interweaving may result in developmental fidelity and robustness of vulval patterning (Braendle and Felix, 2008).

Loss of RAL-1 suppressed the induction of 2° cells by *lin-12(*d*)*; *lin-15(*ts*)* at 16°C, but only partially. We therefore speculate that RGL-1-RAL-1 comprises only part of the pro-2° EGF signal. We note that such a pathway need not be Ras dependent, but is perhaps a different signal transduction pathway engaged directly by activated EGF receptor.

Morphogen gradients have been studied for generations, yet there are still significant mysteries in differential interpretation of signals across gradients

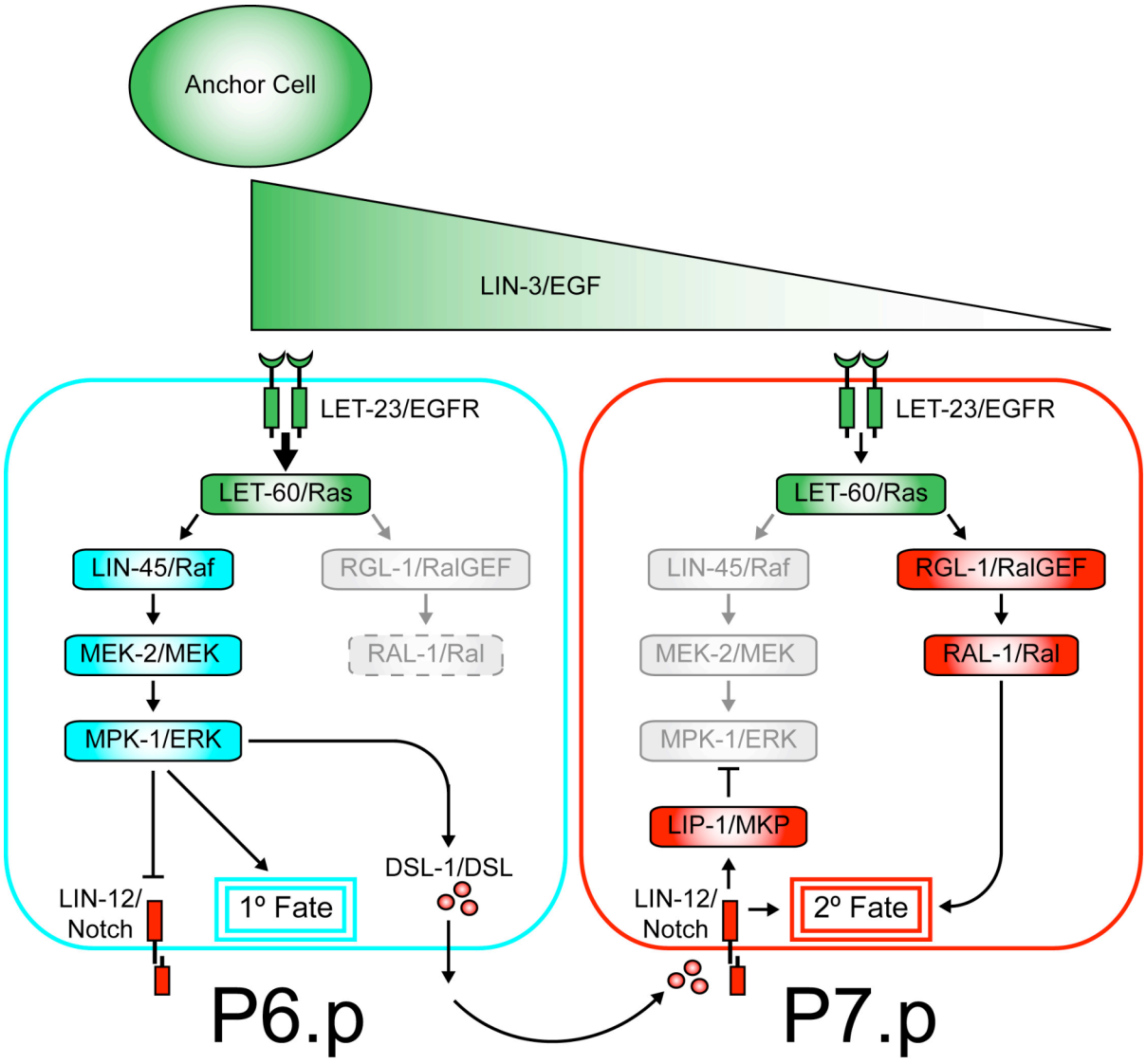


Figure 2-12. EGF Signaling through Ras Uses Effector Switching to Induce Opposing Vulval Fates

Signal promoting both fates is shown in green, pro-1° signal in blue, pro-2° signal in red, and quenched signals in gray. A putative EGF concentration gradient, in combination with sequential induction, faithfully patterns vulval fates. In presumptive 1° cells EGF activates Ras to utilize Raf to promote 1° cell fate. Pro-2° signaling through Notch is quenched. Putative quenching of RGL-1-RAL-1 pro-1° activity is based on RAL-1 exclusion from presumptive 1° cells. Presumptive 1° cells produce DSL ligands to induce neighboring VPCs via Notch to assume 2° fate. In presumptive 2° cells, Notch induces production of LIP-1/ERK phosphatase and other 2°-specific proteins to quench the Raf pro-1° signal. Also, EGF activates Ras to utilize RGL-1-RAL-1 to promote 2° fate. Thus, the EGF signal toggles its developmental output by Ras effector switching.

(Lawrence, 2001). In other systems a variety of gradient response mechanisms exist, from differential transcription of target genes to signal-induced reprogramming of signal response (Ibanes and Izpisua Belmonte, 2008; Piddini and Vincent, 2009), but correlation and causation are not always clear in these systems. In cultured human cells exposed to ectopic EGF or heregulin ligand, downstream pathway utilization varies dramatically by cell line, time of exposure, and ligand concentration (Chen et al., 2009). In vulval patterning EGF gradient input is superimposed on sequential EGF and Notch signals, and our results suggest that Ras effector switching comprises a significant portion of EGF gradient interpretation.

The precise nature of the gradient itself is unclear. Four LIN-3/EGF isoforms have been described, and these have potentially different properties in vulval signaling. Also, the *C. elegans* rhomboid protease ROM-1 influences LIN-3/EGF activity in ways that include differential isoform effects (Dutt et al., 2004; Van Buskirk and Sternberg, 2007). So, work remains to understand the gradient and its interpretation by VPCs.

B. Effector Switching Achieves Divergent Developmental Outcomes from the Same Signal

Our results strongly suggest that Ras switches effector utilization between presumptive 1° and 2° cells. The switching mechanism is unknown, but perhaps Notch signaling reprograms effector use in addition to effector pathway quenching. Additionally, perhaps different EGF concentrations elicit qualitatively distinct signaling activities from EGFR and its pathway. If past studies are an indicator,

there are likely to be multiple overlapping systems that cooperatively reprogram EGF output.

Clearly effector pathway quenching is also critical, since there are significant consequences of loss of LIP-1/ERK phosphatase (Berset et al., 2001). Based on our GFP expression studies we speculate that a similar quenching phenomenon may exist for RAL-1, at least at the transcriptional level, since a *ral-1* reporter is rapidly excluded from presumptive 1° cells after initial induction. RAL-1 quenching occurs in presumptive 1° cells, and is thus complementary to Ras-Raf-ERK quenching in presumptive 2° cells.

Theoretically effector switching can also occur at the level of Ras effector binding. Loss of the *C. elegans* SOC-2/SUR-8 adaptor protein results in diminished Ras-Raf signaling (Selfors et al., 1998; Sieburth et al., 1998). Human SOC-2/SUR-8 physically scaffolds Ras and Raf, thus regulating Ras-Raf association and pathway activation (Li et al., 2000). Although no such protein has been identified for Ras-RalGEF scaffolding, dynamic developmental regulation of such scaffolds could critically impact effector usage.

Previous studies of PC12 pheochromocytoma cell differentiation demonstrated that, via differential effector usage, divergent developmental outcomes arise from a particular signal. Nerve growth factor activation of Ras promotes Raf- and PI3K-dependent neuronal differentiation and growth cessation (Jackson et al., 1996; Sano and Kitajima, 1998). Conversely, Ras activation of RalGEF promotes proliferation and not differentiation (Goi et al., 1999). Thus, Ras has the potential to promote both pro-differentiation and anti-differentiation by engaging different

effectors in the same cell type. In PC12 cells, RalGEF is speculated to be eventually uncoupled from Ras (Goi et al., 1999). Thus, while the potential for effector switching has been demonstrated in cell culture, mechanisms of pathway interaction are lacking.

The cooperative interplay between Notch and Ras signaling in cell fate regulation established in our studies shows striking similarity to a similar Notch and Ras interplay in mouse pancreatic cell differentiation and cancer development (Mysliwiec and Boucher, 2009). Early stage pancreatic cancer development may involve EGFR-mediated, Notch-dependent acinar to ductal cell differentiation (Miyamoto et al., 2003). Concurrent Notch activation cooperates with K-Ras activation to promote acinar differentiation and tumorigenesis (De La et al., 2008). Conversely, Notch inhibition impaired K-Ras-driven pancreatic cancer progression (Plentz et al., 2009). Whether this interplay is dependent on K-Ras activation of the RalGEF-Ral pathway is not known. However, it is intriguing that RalGEF but not Raf is preferentially activated in pancreatic cancer cells and Ral activation is necessary for pancreatic cancer growth (Lim et al., 2005; Lim et al., 2006).

Efforts to develop anti-Ras inhibitors have focused on targeting effector signaling (Yeh and Der, 2007), and have been complicated by cell- and cancer-type differences in effector dependency and activation (Hamad et al., 2002; Lim et al., 2005; Rangarajan et al., 2004; Tuveson et al., 2004). For example, the RalGEF, but not the Raf or PI3K pathway, is activated consistently in the presence of mutationally activated K-Ras in pancreatic cancer. How differential effector utilization and activation is achieved remains an unresolved issue. Our observations establish

mechanisms for this phenomenon that may be at play in cancer cells. Additional mechanisms may involve regulation of the subcellular localization of Ras to distinct membrane compartments, leading to spatial regulation of effector activation (Bivona et al., 2006; Onken et al., 2006).

In conclusion, we demonstrate a patterning role for Ras effector switching that has implications beyond developmental genetics. Studies in model genetic organisms in conjunction with mouse and cell culture studies were instrumental in developing our early understanding of key signal transduction pathways, including canonical EGF signaling through the Ras-Raf-MEK-ERK signaling module to regulate transcription. Recent studies in pathway quenching, and now effector switching, to promote alternative ligand outputs argue that *C. elegans* vulval patterning continues to yield important insights into diverse biological fields.

VI. Acknowledgements

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CHAPTER 3: CONCLUDING REMARKS AND FUTURE DIRECTIONS

Ras is mutated in 30% of all human cancers and as high as 90% in pancreatic cancer. Yet, efforts to develop anti-Ras drugs have to date met with little success. To promote mammalian oncogenesis, Ras signals primarily through the Raf kinase, phosphatidylinositol-3 kinase, and Ral-specific guanine nucleotide exchange factor (RalGEF) downstream effectors. The role of Raf activation of the mitogen-activated protein kinase (MAPK) pathway in Ras-mediated human oncogenesis and induction of *C. elegans* vulval development is well established. Recent studies have defined an important role for RalGEF activation of the Ras-like Ral small GTPases in Ras-mediated oncogenesis and metastasis, but the downstream mechanisms by which this pathway promotes Ras signaling remain poorly defined. I have chosen to focus my dissertation studies on elucidating the *in vivo* developmental role of the RalGEF-Ral pathway in the simple model system *C. elegans.* A controversial model in the vulval development field predicts that EGF signaling in vulval precursor cells, in addition to its well-characterized role in promoting 1° fate via Ras-Raf-MEK-ERK signaling, promotes 2° cell fate directly via an unknown mechanism. In chapter 2, we found that during vulval patterning Ras through Raf transduces a pro-1˚ signal, then through the RalGEF-Ral pathway transduces a pro-2° signal. Our key finding that the Ras-RalGEF-Ral effector pathway promotes 2° cell fate downstream of EGF reconciles current models of *C. elegans* VPC patterning. Furthermore, our study demonstrates the importance of effector switching *in vivo* for re-programming EGF and Ras pathway outputs to achieve divergent developmental outcomes.

I. Does Ras or Other Ras Family Small GTPases Activate the RalGEF-Ral Signaling Module in VPCs?

In mammalian oncogenesis, RalGEF is one of three main downstream effectors of Ras. However, there is evidence in *D. melanogaster* and mammalian cells that other Ras-related proteins, such as R-Ras and Rap, may also activate RalGEFs (Mirey et al., 2003; Spaargaren and Bischoff, 1994; Wolthuis et al., 1997). Since LET-60/Ras signaling through LIN-45/Raf is essential for *C. elegans* vulval development, we could not directly assess the necessity of LET-60/Ras for RGL-1 activation. Thus in Chapter 2, we investigated whether LET-60/Ras activates RGL-1-RAL-1 in VPCs by two indirect methods. First, we utilized animals harboring a putative null mutation ("0") in *lin-31, a* winged helix domain transcription factor (TF) downstream of LET-60/Ras-LIN-45/Raf signaling. This mutation causes a moderate hyper-induced vulval phenotype thought to be Raf-MEK-ERK-independent. Loss of *let-60*/Ras, but not *lin-45*/Raf or *mpk-1*/MAPK, enhanced the *lin-31(*0*)* hyper-induced phenotype, suggesting that LET-60 also transduces a signal that antagonizes the pro-1° LET-60/Ras-LIN-45/Raf signal. Second, we generated constitutively active LET-60/Ras additionally mutated to be specific for RalGEF effector usage (LET-60(12V, 37G)). We found that VPC-directed expression of LET-60(12V, 37G) suppressed the hyper-induced phenotype of *let-60(*gf*)* animals in a *rgl-1*- and *ral-1*-dependent manner. This suppression was equivalent to the effect of RAL-1(gf), and suggested that LET-60/Ras induces RGL-1-RAL-1 activity.

These two experiments, however, are subject to several caveats. First, though the hyper-induced phenotype of *lin-31(*0*)* is LET-60/Ras-LIN-45/Raf

independent, it may not provide the best background for evaluating putative LET-60-RGL-1-RAL-1 signaling. When inactive, LIN-31 forms a complex with LIN-1, an ETS-domain TF. Phosphorylation of LIN-31 by the MPK-1 ERK MAPK disrupts this complex and stimulates vulval fates (Tan et al., 1998). However in *lin-31* mutants, VPCs randomly adopt vulval (1° or 2°) or nonvulval (3°) fates (Miller et al., 1993). Thus, LIN-31 appears to stochastically regulate VPC fates, complicating the analysis of proteins that regulate a certain fate (e.g. pro-2° proteins). Second, over-expression of mutationally-activated small GTPases may not accurately model EGF activation of wild-type GTPases, and may additionally be mislocalized, leading to permissive effector binding. Thus, over-expression of LET-60(12V, 37G) could cause artifactual activation of RGL-1. Since LET-60/Ras activation of the RGL-1-RAL-1 pathway is the central premise of our "effector switching" VPC patterning model, it will be important to further test whether LET-60/Ras directly activates RGL-1-RAL-1.

An alternative *in vivo* functional approach that would aid in teasing apart the LET-60/Ras effector pathways and circumvent the lethality of loss of LET-60/Ras is to activate LIN-45/Raf in a Ras-independent manner. We could generate an activated LIN-45/Raf through mutation of the RBD (to prevent Ras binding) together with the addition of the LET-60/Ras C-terminal plasma membrane targeting sequence. This approach has been previously utilized to generate a constitutively activated variant of human c-Raf-1 (Leevers et al., 1994). This construct should induce a Muv phenotype in a WT background that is independent of LET-60/Ras signaling. Since our data suggest that RGL-1 signaling acts through a pathway

parallel to LIN-45/Raf, *rgl-1*- and *ral-1*-directed RNAi should enhance this Muv phenotype. If LET-60/Ras signals through RGL-1, then we predict that loss of *let-60* by RNAi or hypomorphic mutation should result in enhancement of the Ras-independent LIN-45/Raf phenotype.

Yeast-two-hybrid experiments, though potentially artifactual, have found that activated LET-60/Ras binds RGL-1 (Shibatohge et al., 1998). Biochemical analysis with authentic full length proteins could be performed to confirm the interaction of these two proteins. Cell expression-based glutathione-*S*-transferase (GST)-pulldown assays could be used to determine if LET-60/Ras is capable of binding RGL-1 in a GTP-dependent manner, and if this binding leads to RAL-1 activation.

It is also possible that a GTPase other than LET-60/Ras activates RGL-1 in the VPCs. There are four other *C. elegans* Ras family small GTPases that could potentially bind to the RA domain and activate RGL-1: RAS-1/R-Ras, RAS-2/M-Ras, RAP-1/Rap, and RAP-2/Rap. It is predicted that loss-of-function of the upstream activator will result in the same enhancement of the Muv phenotype as loss-of-function of RGL-1. Thus, loss-of-function analysis (RNAi or mutation) in the *let-60(*gf*)* background could be performed to determine whether any of these alternate small GTPases activate RGL-1. We have previously tested *rap-1(RNAi)* and *rap-2(RNAi)* and found that neither altered the *let-60(*gf*)* phenotype alone. However, it is possible that the two Rap orthologs are acting redundantly in the VPCs. To address redundancy, pairwise knockdowns of candidate RGL-1 activators could be performed using fRNAi and null deletion alleles. Since loss of both RAP-1 and RAP-2 is known to cause early larval lethality, we will need to analyze the double loss-of-

function only in the VPCs (Pellis-van Berkel et al., 2005). To circumvent the larval lethality, we could introduce *rap-1*-directed hairpin RNAi driven specifically in the VPCs into the *let-60(n1046*gf*); rap-2(gk11*lf*)* double mutant background. If Rap is a putative RGL-1 activator, than this VPC-specific double Rap knockdown should result in enhancement of the *let-60(n1046*gf*)* hyper-induced phenotype.

II. Which Downstream Effectors of RGL-1-RAL-1 Mediate the Effect of Ral on Vulval Development?

Several Ral effectors and binding proteins have been identified biochemically, and little is known about their *in vivo* relationship to Ral function. Since RalGEF-Ral signaling is important in Ras-mediated transformation of human cells, understanding how RalGEF-Ral signaling is propagated may provide insight into Ral-mediated tumorigenicity and Ral isoform differences. An important future direction will be to further elucidate the RAL-1 effector pathways important in VPC patterning. In chapter 2, we utilized RNAi to evaluate the role of putative *C. elegans* RAL-1 effector orthologs in VPC patterning. We found that RNAi of three putative RAL-1 effectors, RLBP-1/RalBP1, Y66H1B.3/Filamin, and PLD-1/ PLD, enhanced the *let-60(*gf*)* hyper-induced phenotype. However, RNAi of no single effector was quantitatively equivalent to suppression of RGL-1 or RAL-1 activity, suggesting that multiple effectors function cooperatively downstream of RAL-1 in vulval patterning. One important caveat to this experiment is that RNAi does not always work with equal efficacy on different genes. To address this issue, we could utilize the *eri-1(mg366)* RNAi hypersensitive mutant strain. The *eri-1(mg366) let-60(n1046*gf*)* strain is

predicted to be more responsive to pathway RNAi than *let-60(*gf*)* alone. Thus this strain may be more sensitive to RNAi of putative RAL-1 effectors. We could also analyze null alleles for putative RAL-1 effectors instead of using RNAi. Null RAL-1 effector alleles would also allow us to analyze loss of RAL-1 effectors in different combinations to test effector redundancy.

To further evaluate the putative RAL-1 effectors, we could also utilize activated RAL-1(Q75L) with missense effector domain mutations that are predicted to differentially abolish the binding of a subset of Ral effectors. Such reagents have been characterized in human cells (Moskalenko et al., 2002). We should be able to generate equivalent functional mutations in RAL-1, since the effector domain sequences of *C. elegans* RAL-1 are 100% conserved with human Ral orthologs. For example, we could generate an activated RAL-1(Q75L) plus a deletion of the first 11 amino acids, which is predicted to abolish PLD-1 signaling. If PLD-1 is the relevant vulval effector then this effector domain mutation should rescue the RAL-1(Q75L) suppression of the *let-60(*gf*)* hyper-induced phenotype. However, if PLD-1 is not a critical RAL-1 effector in the VPCs, then this mutation should still suppress the *let-60(*gf*)* hyper-induced phenotype. Two other Ral effector domain mutations have been characterized in human cells and could be generated in RAL-1: the 49E mutation (RAL-1 equivalent 52E) signals through RalBP1 but not the exocyst complex (Sec5/Exo84), and the 49N mutation (RAL-1 equivalent 52N) signals through the exocyst but not RalBP1.

It is also possible that unidentified Ral effector orthologs function downstream of RAL-1 in VPC patterning. We could utilize the activated RAL-

1(Q75L) background to screen for candidate RAL-1 effectors. RAL-1(Q75L) suppresses the Muv phenotype of *let-60(*gf*)*, and this suppression should be dependent upon the activity of RAL-1 effectors. Therefore, RNAi of a candidate Ral effector should abolish the activated RAL-1 suppression, and the resulting phenotype should resemble that of *let-60(*gf*); ral-1(RNAi).* Alternatively, epitope-tagged constitutively activate RAL-1(Q75L) could be utilized to pull down proteins that bind specifically to GTP-bound Ral. Mass spectrometry techniques could then be used to identify the binding proteins.

III. What Signals Regulate RAL-1 Expression?

In chapter 2, we analyzed a *ral-1* promoter-driven GFP fusion construct to determine the RAL-1 expression pattern during vulval development. We found that RAL-1 is dynamically expressed in the VPCs. Initially RAL-1 is expressed in all VPCs but is rapidly restricted, first to EGF-induced VPCs and then to presumptive 2° cells. We consider RAL-1 exclusion from presumptive 1° cells immediately post-induction to be a form of signal quenching, which restricts the RAL-1 pro-2° signal to presumptive 2° cells. One caveat to this approach for determining the RAL-1 expression pattern is that the DNA segment fused may not contain all the elements required to accurately control transcriptional expression of the endogenous gene product. The genome of *C. elegans* is relatively densely packed (1998). Thus, a DNA fragmConsortiument from immediately upstream of the protein-coding region tends to contain the promoter and the majority of the transcriptional regulatory elements. However, our reporter fusion will of course lack all post-transcriptional

aspects of regulation. It may also lack transcriptional regulatory elements located in introns, exons, or the 3' UTR. In an alternative approach, we could generate the full-length *ral-1* region including 4 kb of promoter and 1 kb of 3' sequences, and utilize recombineering technology to insert GFP in-frame after the transcription initiation sequence (Myers and Greenwald, 2005).

The restriction of RAL-1 expression to presumptive 2° cells may elucidate part of the mechanism by which LET-60 effector usage is changed in presumptive 2° cells. Thus, an important future direction will be to elucidate what signals regulate the expression of RAL-1 in the VPCs. The early vulval RAL-1 expression pattern mirrors that of LIP-1 (Berset et al., 2001). The restriction of RAL-1 expression to presumptive 2° cells, similar to LIP-1, suggests that TFs specifically activated in presumptive 2° cells may regulate the expression of RAL-1. LIN-12/Notch is known to regulate the expression of several pro-2° proteins including LIP-1. Thus, it is possible that LIN-12/Notch also induces presumptive 2° specific expression of RAL-1. However, in previous computational analyses, RAL-1 was not identified as a LIN-12/Notch target (Yoo et al., 2004). Furthermore, we have not found LIN-12/Notch responsive elements in the *ral-1* promoter. Alternatively, RAL-1 expression may be regulated by the LIN-45/Raf effector pathway, or by a RGL-1-RAL-1 feedback loop. To determine what signals regulate RAL-1 expression, we could assess the effect of gain-of-function or loss-of-function mutations in pro-1° or pro-2° pathways on $P_{ral\text{-}1}$*::gfp* expression. For example, if LIN-12/Notch regulates RAL-1 expression, then we would expect RAL-1 to be expressed in all VPCs in a *lin-12(*gf*)* background. In

this manner we could test known pro-1° and pro-2° pathway components for their effects on RAL-1 expression.

IV. Does RGL-1-RAL-1 Signaling Play a Role in Other *C. elegans* Tissues Specified by LET-60?

Elucidating the developmental role of RalGEF-Ral may aid in understanding how cancer hijacks normal developmental programs to promote oncogenesis. To further characterize the role of RGL-1 and RAL-1 in *C. elegans,* we obtained several knockout mutants from the *C. elegans* knockout consortium. We worked with three different deletion alleles of *rgl-1,* all of which are likely nulls for RAL-1 activation. *rgl-1 (gm27)* is a small deletion of the X chromosome that removes *rgl-1* and eight additional genes, making this a suboptimal reagent (Much et al., 2000). *rgl-1(tm2255)* and *rgl-1(ok1921)* delete the CDC-25 GEF homology region, and thus should fail to activate RAL-1 nucleotide exchange. *rgl-1(tm2255)* also disrupts the subsequent reading frame, while *ok1921* is an in-frame deletion. We constructed double mutant strains of *let-60(n1046*gf*)* with each *rgl-1* deletion allele, but observed high lethality levels. Double mutant animals, particularly at later larval stages, rupture at a point ventral to the pharynx. About 75% of animals that survive display a protrusion at this location (Figure 3-1). This high lethality hampered our ability to accurately score vulval induction, since the majority of animals rupture before larval stage 4.

Similar cuticle protrusion defects have been observed in *let-60(n1046*gf*)* single mutants at a much lower frequency (~10%) (Yochem et al., 1997). Cuticle

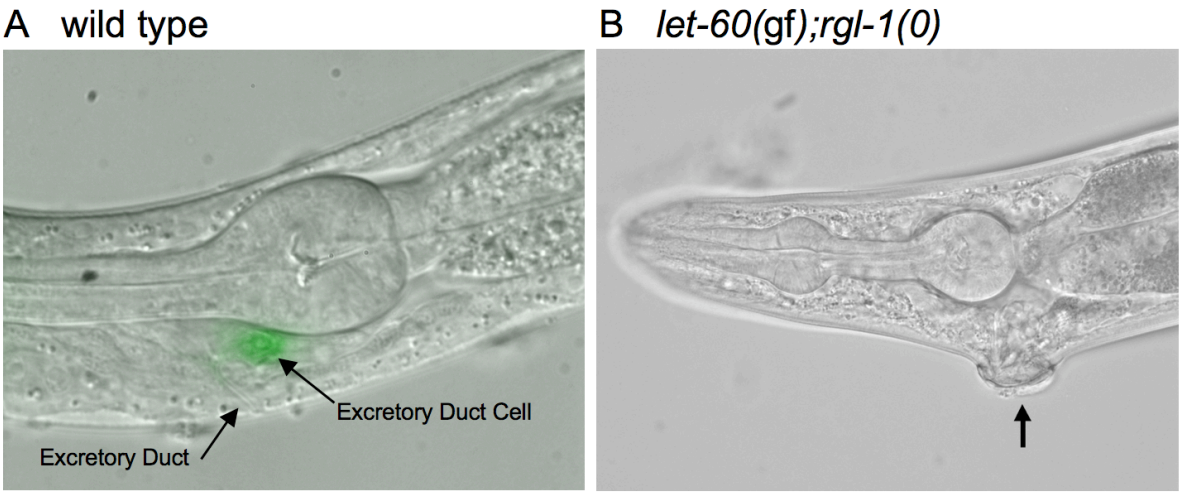

Figure 3-1. Role of RGL-1 in Excretory Duct Cell Specification.
(A) DIC micrograph of the excretory duct cell in a wild type L3 larvae expressing P_{lin-48}*::gfp* (excretory duct cell reporter) **(B)** DIC micrograph of *let-60(n1046*gf*); rgl-1(tm2255)* as late L3. Many double mutant animals rupture at a point ventral to the pharynx. About 75% of surviving animals display a protrusion at this location (marked by an arrow). It is possible that this cuticle protrusion is due to duplication of the excretory duct cell.

protrusions ventral to the posterior bulb of the pharynx can result from duplication of the excretory duct cell or the excretory pore cell, components of the osmoregulatory system (Lambie and Kimble, 1991; Nelson and Riddle, 1984; Yochem et al., 1997). Like specification of 1˚/2˚ fates in the vulva, specification of the excretory duct cell is a binary cell fate decision controlled by the EGFR-Ras-Raf-MAPK pathway. Ablation experiments suggest that during embryonic development the AB.pl and AB.pr lineage form an equivalence group whose specification requires both Ras and Notch signaling (Sulston et al., 1983; Yochem et al., 1997). In a wild-type animal, the AB.pl descendant differentiates into the duct cell, and the AB.pr descendant generates the G1 cell that forms the excretory pore. In a *let-60(*gf*)* background animals often develop two duct cells, whereas in a *let-60(*lf*)* background animals suffer early lethality due to lack of a duct cell.

We found that *let-60(*gf*);rgl-1(0)* double mutants display increased cuticle protrusions ventral to the posterior bulb when compared to either single mutant alone (data not shown). It is possible that these protrusions are due to increased duct cell duplications. We speculate that, much as in vulval induction, RGL-1 is acting to antagonize LET-60 function during excretory duct induction. Thus the loss of RGL-1 results in enhancement of LET-60 signaling leading to duplication of the excretory duct cell. Further analysis with an excretory duct cell marker, $P_{lin\text{-}48}$*::gfp* needs to be performed to determine whether these ventral pharyngeal protrusions are actually due to excretory duct cell duplications.

In chapter 2 we analyzed a RAL-1 deletion allele *(tm2760).* We found that deletion of RAL-1 significantly enhanced the hyper-induced phenotype of *let-60(*gf*)*.

However, while analyzing this strain, we did not notice a significant increase in ventral pharyngeal protrusions (data not shown). Thus, it is possible that RGL-1 performs a function in excretory duct cell specification other than RAL-1 activation. To test this possibility, we could transgenically rescue the RAL-1-independent functions of RGL-1 by expressing a RGL-1-expressing construct in which a point mutation renders the CDC-25 catalytic exchange activity dead (R446E), while the rest of the protein is intact (Lim et al., 2005). If RGL-1 regulates specification of the excretory duct cell via RAL-1-independent mechanisms, then expression of catalytically dead RGL-1 should suppress the duct cell duplication phenotype, whereas expression of RAL-1(gf) should fail to rescue.

V. Is RAL-1 More RalA- or Ral B-like?

There is considerable evidence that the highly related RalA and RalB isoforms serve divergent roles in Ras-mediated oncogenesis. However, the mechanistic basis for their distinct roles has not been established. Based on amino acid sequence alignment alone, it is difficult to determine if RAL-1 is more RalA-like or RalB-like. An important future direction will be to determine if RAL-1 is functionally conserved with RalA and/or RalB. This knowledge may aid in extrapolating RAL-1 findings in *C. elegans* to mammalian development.

HEK-HT (human embryonic kidney) cells that ectopically express SV40 T-ag, SV40 t-ag, and hTERT have previously provided a useful model to evaluate Ral-mediated growth transformation (Figure 3-2) (Hamad et al., 2002). Thus this cell line may provide a useful mammalian cell model to determine whether RAL-1 is

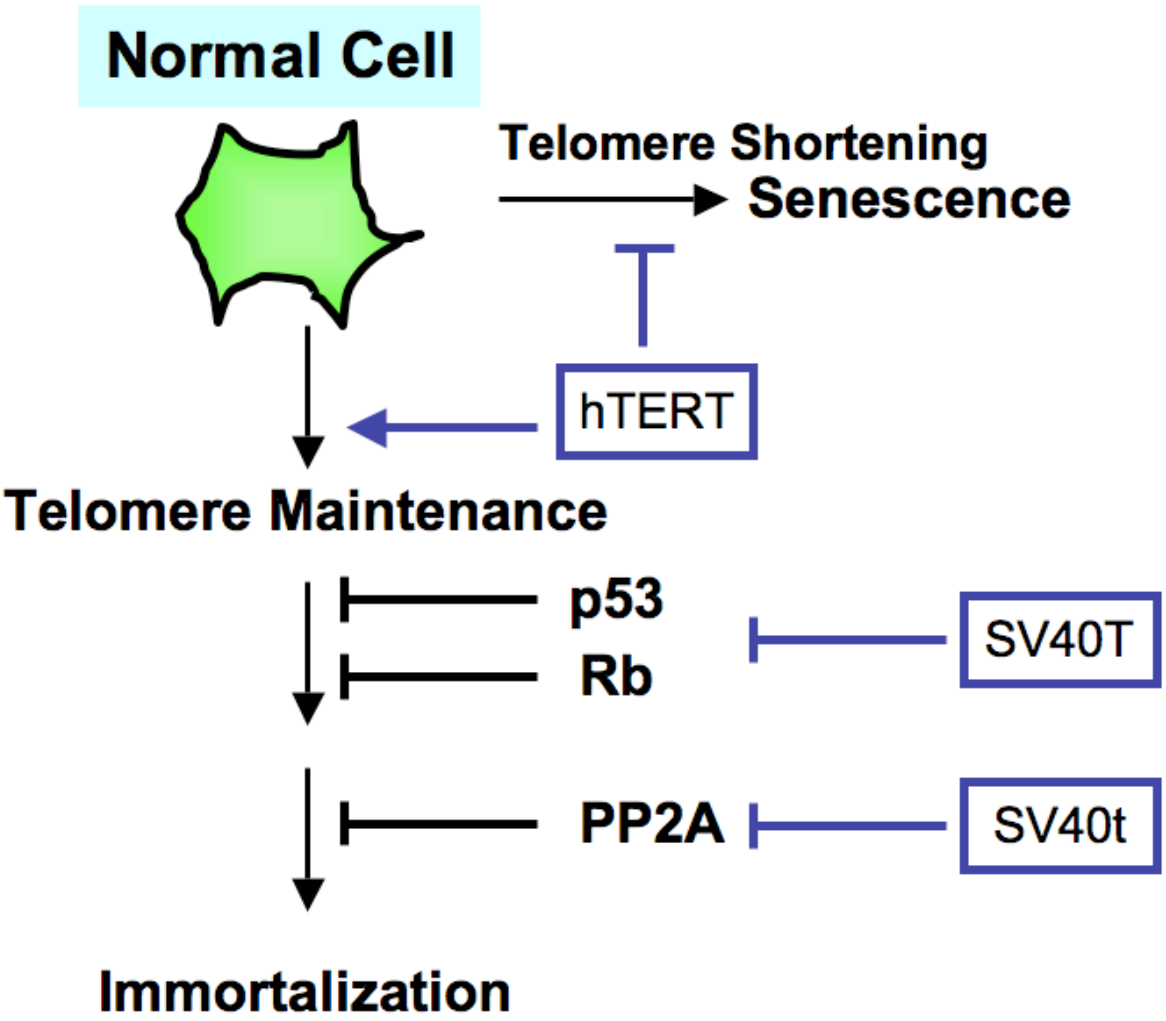

Figure 3-2. Immortalization of Human Cells.
Ectopic expression of *hTERT* and the early region of *SV40* can immortalize normal human cells. The *hTERT* gene encodes the catalytic subunit of the telomerase haloenzyme and acts to promote telomere maintenance by inhibiting telomere shortening (Bodnar et al., 1998; Counter et al., 1998; Vaziri and Benchimol, 1998). The *SV40* early region encodes both the large T and small t antigens. SV40 T-ag disrupts the functions of the tumor suppressors p53 and Rb (Livingston, 1992; Ludlow, 1993). The SV40 t-ag disrupts the function of the tumor suppressor phosphatase PP2A (Pallas et al., 1990; Rubin et al., 1982).

functionally conserved. To determine functional conservation, HEK-HT cells expressing RAL-1(Q75L) could be assayed for anchorage-independent growth when suspended in soft agar (Cifone and Fidler, 1980). The soft agar assay is one of the most stringent tests of cellular transformation, and provides an *in vitro* correlate to tumorigenic growth potential. The migration ability of these cells could also be evaluated using the trans-well migration assay to assess migratory potential (Oxford et al., 2005). Additionally, HEK-HT cells expressing GFP-tagged constitutively activated RAL-1 could be visualized to determine the subcellular localization of RAL-1. If RAL-1 is functionally conserved with human Ral isoforms, then we predict constitutively active RAL-1(Q75L) to be localized primarily to the plasma membrane. If RAL-1 is more RalB-like, we may also observe localization to recycling endosomes. If *C. elegans* RAL-1 is more RalA-like, then cells expressing activated RAL-1(Q75L) are predicted to support anchorage-independent growth, but display little or no migration. If *C. elegans* RAL-1 is more Ral-B like, then cells expressing activated RAL-1(Q75L) are predicted to not support anchorage-independent growth, but display increased migration.

Alternatively, we could determine whether human RalA and/or RalB recapitulate RAL-1 effects in *C. elegans* vulval development by expressing constitutively activated RalA(Q72L) or RalB(Q72L) specifically in the VPCs of *let-60(*gf*)* animals. If human RalA and/or RalB can recapitulate *C. elegans* RAL-1 effects in vulval induction, expression of RalA(Q72L) and/or RalB(Q72L) constructs would be expected to suppress the *let-60(*gf*)* hyper-induced phenotype similar to the suppression seen with activated RAL-1(Q75L). It is possible that both, one, or

neither isoform will duplicate the RAL-1 phenotype. If both isoforms cause the same phenotype as activated RAL-1, then perhaps the subtle localization differences that lead to functional differences in mammalian cells are either not relevant in *C. elegans* or are over-ridden by ectopic over-expression. Only one isoform recapitulating RAL-1 effects suggests that isoform is the functionally conserved protein. If the human isoforms have diverged too much, then neither isoform may functionally replace RAL-1. Instead of ectopically over-expressing activated RalA or RalB, we could perform rescue experiments. In this approach, we could determine whether expression of wild type RalA and/or RalB can rescue the loss of RAL-1.

VI. Is the Interplay between LET-60-RGL-1-RAL-1 and LIN-12 Conserved in Mammals?

Several studies have found that the Ras and Notch pathways sometimes cooperate and sometimes antagonize each other (Sundaram, 2005). The differences leading to cooperation or antagonism are poorly understood. In *C. elegans* vulval development, a combination of Ras-Raf and Notch signaling leads to divergent cell fates. It is well characterized that the Ras-Raf pathway promotes 1° cell fate, whereas the Notch pathway promotes 2° cell fate (Sternberg, 2005). Also, within a given VPC, the Ras and Notch pathways activate methods to antagonize each other (Berset et al., 2001; Shaye and Greenwald, 2002). While Ras-Raf antagonizes the pro-2° Notch signal in the P6.p, in chapter 2 we found that Ras-RalGEF-Ral cooperates with Notch in the adjacent P5.p and P7.p to specify 2° cell fate. Thus, depending on the Ras effector pathway utilized, Ras and Notch can both

cooperate and antagonize each other. An important future direction will be to test whether Ras effector utilization impacts its relationship with Notch signaling in mammals.

Rat pheochromocytoma PC12 cells are a model system for differentiation of neuronal cells, and may provide a good mammalian model system to analyze the complex interplay between Ras and Notch signaling pathways. Previous studies in PC12 cells have demonstrated that different cellular outcomes can arise from the same signal via differential effector usage. In this model system, nerve growth factor (NGF) activation of Ras promotes Raf- and PI3K-dependent growth cessation and terminal differentiation into a neuronal phenotype (Jackson et al., 1996; Sano and Kitajima, 1998; Wood et al., 1992). Conversely, Ras activation of RalGEF suppresses neurite outgrowth by promoting proliferation (Goi et al., 1999). Thus, Ras has the potential to promote both pro-differentiation and anti-differentiation through differential activation of distinct effector pathways. Notch signaling has also been found to suppress NGF-induced neurite outgrowth. However, whether the Ras-RalGEF-Ral and Notch signaling pathways cooperate to suppress neurite outgrowth is not known. To test whether these two pathways cooperate, we could utilize RNAi against RalGEF or Ral in PC12 cells expressing constitutively active Notch. If RalGEF and Notch cooperate, then RNAi-mediated knockdown of RalGEF or Ral would be expected to enhance NGF-induced neurite outgrowth. The reciprocal of this experiment could also be performed: Notch RNAi-mediated knockdown in PC12 cells expressing constitutively active RalGEF or Ral.

Several studies have also suggested that Ras and Notch can cooperate to promote oncogenesis (Yeh and Der, 2007). The mechanisms by which these two pathways cooperate, however, remain unclear. One important future direction will be to determine whether the cooperation between the Ras and Notch pathways is dependent on the RalGEF-Ral effector pathway. Dominant negative Ral or RNAi directed against RalGEF or Ral could be utilized to determine whether Notch-mediated tumor cell lines are dependent on RalGEF-Ral for anchorage-independent growth. Previous studies have found that activation of Ras upregulates the expression of some Notch ligands (Chen and Greenwald, 2004; Weijzen et al., 2002). Therefore, it will be important to test whether constitutively active Ral upregulates the expression of Notch ligands and whether this leads to increased levels of activated Notch (NICD).

VII. Does the Quantitative Strength of Ras Pathway Activation Result in Differential Effector Utilization in Mammals?

In *C. elegans*, LIN-3/EGF activation of the EGFR-Ras-Raf pathway to promote the 1° cell fate is well characterized. Additionally, an anchor cell-centered LIN-3/EGF gradient has been shown to exist, and LIN-3/EGF is sufficient to promote 2° fate (Katz et al., 1995; Yoo et al., 2004). In chapter 2, we utilized temperature to titrate LIN-3/EGF levels in the VPCs. Though high levels of LIN-3/EGF promote the 1° cell fate (presumably through Ras-Raf), we found that low levels of LIN-3/EGF promote the 2° cell fate in a RAL-1-dependent manner. Thus different levels of pathway activation may trigger differential effector utilization to achieve divergent

cellular outcomes. Several studies have found that different levels of Ras activation can elicit differential biological outcomes (Greenwood and Struhl, 1997; Murphy et al., 2002; Sabbagh et al., 2001). However, whether different levels of Ras activation result in differential effector usage remains poorly understood. An important future direction will be to determine whether Ras effector switching can occur in response to the quantitative strength of pathway activation in mammals.

In PC12 cells, previous studies have identified that EGF produces transient low levels of Ras activation (as measured by GTP-bound Ras) that trigger proliferation, whereas NGF produces sustained high levels that trigger differentiation (Huff et al., 1981; Muroya et al., 1992; Nguyen et al., 1993; Traverse et al., 1994). It is thought that the divergent outcomes arise solely from sustained ERK activation (Marshall, 1995). Our observations, when considered together with the recent identification of the opposing roles of RalGEF and Raf in neurite outgrowth, suggest that different levels of pathway activation (mediated by NGF and EGF) may result in differential Ras effector utilization. Based on previous studies, we would expect EGF-induced PC12 cells to have low levels of phosphorylated ERK when compared to NGF-induced PC12 cells (as assessed by immunoblot with an ERK phosphospecific antibody). Similarly, we could assess Ral activation levels in PC12 cells induced by EGF or NGF. We could utilize a glutathione-*S*-transferase (GST) fusion protein containing the Ral-binding domain of the Ral effector protein RalBP1 to isolate active Ral (GTP-bound Ral) followed by immunoblot analysis (Wolthuis et al., 1998). We expect that EGF induction would result in more robust elevation of the levels of Ral-GTP in comparison to NGF induction.

In summary, my studies establish Ras effector switching as one mechanism by which the EGF signal promotes divergent developmental outcomes in adjacent cells. The high mutation frequency of Ras in human cancers has led to extensive analysis of Ras effector pathways to identify useful targets for anti-Ras therapies. However, targeting Ras in cancer has to date been unsuccessful. One major problem is that cancer cells depend on different effector pathways, and thus respond differentially to effector-targeted pharmacologic therapeutics. Since Ras effectors are largely expressed ubiquitously, understanding how Ras effector utilization is regulated may be key to targeting Ras effector pathways in cancer. Our observations may provide one explanation for why some cancer cells depend on different effector pathways, and respond differentially to effector-targeted pharmacologic therapeutics currently under clinical evaluation. Future studies that better establish the mechanisms and cellular consequences of differential Ras effector usage will likely lead to improved therapeutics for targeting Ras-driven cancers.

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